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EFFECTS OF INCUBATION TEMPERATURE AND STORAGE PERIOD ON FLAVOR AND AROMA COMPOUNDS IN KEFIR

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ABSTRACT

Kefir is fermented milk that has a unique flavor and aroma. The aim of this study was to determine the effects of incubation temperature and storage period on flavor and aroma compounds of kefir. Kefir starter was added into pasteurized and homogenized milk with 2.5% fat and the samples were incubated at 30, 33 and 35ºC. Flavor and aroma compounds (acetaldehyde,diacetyl, acetoin and ethanol) and microbial counts of kefir samples were determined on days 1, 7 and 14 during cold storage. Levels of acetaldehyde, diacetyl, acetoin and ethanol increased with raising of temperature and decreased significantly ($p<0.05$) during cold storage from first day to 14th day. Microbial population (Yeasts, lactobacilli and lactococci) increased significantly ($p<0.05$) with raising of temperature and decreased significantly ($p<0.05$) during 14 days of cold storage.

Keywords: Flavor and aroma compounds, Kefir, Incubation temperature, Storage period

INTRODUCTION

Kefir is fermented milk that has acidic flavor, yeasty aroma, creamy consistency and slightly alcoholic odor (Witthuhn *et al*., 2005). Kefir is made by inoculating milk with kefir grains. Kefir grains are like cauliflower blossoms, yellowish-white, small and hard. They are made of every kind of milk: cow, goat, sheep, camel, buffalo, coconut, rice and soybean; but it is common to use cow milk (Otles and Cagindi, 2003). There is a complex mixture of lactic acid bacteria, Acetobacter and yeast species in kefir (Piermaria *et al.*, 2008; Plessas *et al*., 2008). Kefir microbial population produces lactic acid and other metabolites that increases milk maintenance potentiality and prevents growth of pathogenic microorganisms (Witthuhn *et al.,* 2005).

Kefir unique flavor is because of lactic acid, ethanol, carbon dioxide and other flavor products, such as acetaldehyde, acetoin and diacetyl (Beshkova *et al*., 2003). Production of vitamin B1, B12, calcium, amino acids, folic acid and vitamin K increases during fermentation (Irigoyen *et al.,* 2005).

The objective of this study was to determine the effect of incubation temperature and storage period on flavor and aroma compounds of kefir.

MATERIALS AND METHODS

1. Materials

Kefir starter culture was purchased from Sacco company. DVS starter made by Sacco (Lyofast MT 030 N) included *Lactococcus lactis* subsp. *lacis, Lactococcus lactis* subsp. *lactis biovar diacetylactis, Lactobacillus brevis,* and *Saccharomyces cerevisiae.*

Standards include acetaldehyde and diacetyl were purchased from Sigma Chemical Co, acetoin from Fluka and finally ethanol from Merck Chemical Co.

2. Kefir sample preparation

Sacco package was 10 Unit and it was suitable for 1000 L milk. It was added into 1 L milk and vortexed for 30-45 min (150 rpm). Four ml of mixture of milk and starter was added into 4 L pasteurized and homogenized milk with 2.5% fat. Immediately after inoculation, milk was incubated at one of temperatures (30, 33 and 35 ºC).The desired final pH of the product was 4.6. After that, samples were transferred to refrigerator and stored for two weeks at 4ºC. Physicochemical and microbial experiments were done on days 1, 7 and 14 during cold storage

3. Determination of pH and acidity

pH was measured by direct measurement with a pH-meter (120 Corling, USA) and titratable acidity was measured by AOAC method (2002).

4. Microbial analysis

Kefir samples for counts of lactobacilli and lactococci were plated on MRS agar and Azid agar (Merck Co.), respectively (Atlas, 2006). Both cultures were incubated for 3 days at 30 °C. Malt extract agar (Merck Co.) was used for counts of yeasts. Samples were incubated for 3 days at 25°C (Atlas, 2006). Microbiology count data were expressed as log of colony forming units per ml of soymilk kefir (cfu/mL).

5. Determination of flavor and aroma compounds

Twenty grams of each sample was diluted with 30 mL distilled deionized water, HCl was added until the pH decreased to 2.5, vortexed for 1h at 25 ºC. Samples were left for 2h to coagulate, centrifuged for 10 min (4000 rpm) to separate coagulations, collected upper solutions and defatted with normal hexane. They were passed through Sephadex column (G 75 – 15*1 cm) to remove proteins and other polymers (Dean, 1974). Again, samples were passed through XAD-2 column (10cm*1cm) to remove sugars and non-polar compounds (Grabarczyk and Korolczuk, 2010). Passed solution was extracted with 15 mL diethyl ether two times and cooled at 0 ºC.

For the volatile component analysis, 8 mL sample was transferred into GC vials, injected onto a 3 m Propac Q column (1.6 inch diameter) maintained at 100 ºC. The column temperature programmed at 150 ºC and temperature of FID detector was 250 ºC. Argon (flow of 20 mL/min) was used as the carrier gas (Determann, 1972).

Standard solutions of acetaldehyde, acetoin, diacetyl and ethanol were prepared with distilled deionized water. To remove error, standard addition was done for all samples and analysis was repeated. Qualification of the volatile components in the experimental samples was accomplished by comparison between retention time of samples and standard solutions**.**

6. Statistical analysis

The data were analyzed using two-way analysis of variance (ANOVA) by SPSS 16. Duncan′s multiple range test was used to compare the means when a significant variation was established by ANOVA at the significance level 0.05(α= 0.05). All samples were analyzed in three times.

RESULTS AND DISCUSSION

Changes of acidity in kefir samples are shown in Fig**. 1**. Moreover, Changes of pH in the samples are shown in Fig .**2**. Acidity increased significantly ($p<0.05$) with raising of temperature and increased during cold storage ($p<0.05$) pH decreased significantly ($p<0.05$) with raising of temperature and decreased during cold storage ($p<0.05$). Guzel–Seydim *et al*., (2005) found similar results. They reported that organic acids may occur in dairy products as a result of hydrolysis of fatty acids, biochemical metabolic processes, or bacterial metabolism. Acidity increased when temperature or time of storage was increased. Production of lactic acid was slow at first and then it would be fast. The amount of lactic acid is related to growth of lactic acid bacteria.

Acetaldehyde concentration increased significantly ($p<0.05$) with raising of temperature and decreased during cold storage ($p<0.05$). The most amount of acetaldehyde was only o.933 µg/g in sample produced at 35 ºC first day and the least was 0.34 µg/g in sample produced at 30 ºC th day (Fig. **3**). Beshkova *et al.,* (2003) showed that acetaldehyde concentration decreased during cold storage at 7 days. Acetaldehyde can be converted to ethanol by alcohol dehydrogenase (Tamime and Robinson, 1983).

In all samples, ethanol concentration increased significantly ($p<0.05$) with raising of temperature and decreased during cold storage ($p<0.05$). The most amount of ethanol was 1863.3 µg/g in sample produced at 35 ºC first day and the least was 405/6 µg/g in sample produced at 30 ºC th day (Fig. 4). Higher alcohol content may be associated with a yeasty flavor and, in fact, authentic kefir does have a very slight yeasty flavor (Vedamuthu, 1977).

Acetoin concentration increased significantly ($p<0.05$) with raising of temperature and decreased during cold storage ($p<0.05$). The most amount of acetoin was 0.396 µg/g at 35 ºC first day and the least was 0.050 µg/g at 30 ºC th day (Fig. **5**). Beshkova *et al.*, (2003) showed that acetoin concentration decreased during cold storage at 7 days. Production of acetoin by yeasts is stimulated by acetaldehyde addition (Chuang and Collins, 1968).

Diacetyl concentration increased significantly ($p<0.05$) with raising of temperature and decreased during cold storage ($p<0.05$). The most amount of diacetyl was 0.168 µg/g at 35 ºC first day and the least was 0.032µg/g at 30 ºC th day (Fig. **6**). Beshkova *et al.*, (2003) showed that diacetyl concentration decreased during cold storage at 7 days.

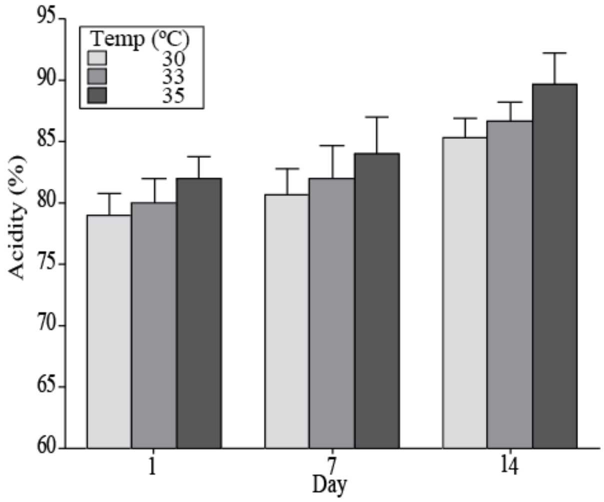

Fig.1. The effect of incubation temperature and storage period on acidity of Kefir samples.

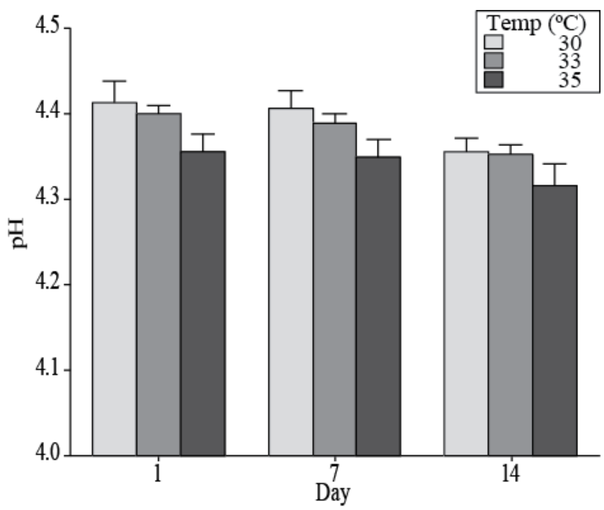

Fig.2. The effect of incubation temperature and storage period on pH of Kefir samples.

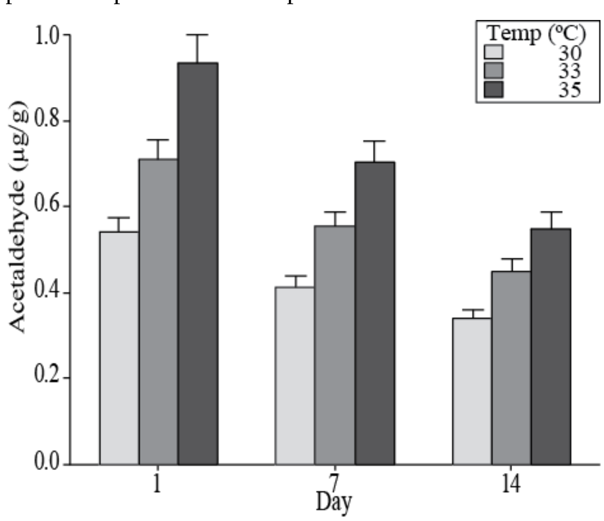

Fig.3. The effect of incubation temperature and storage period on acetaldehyde of Kefir samples.

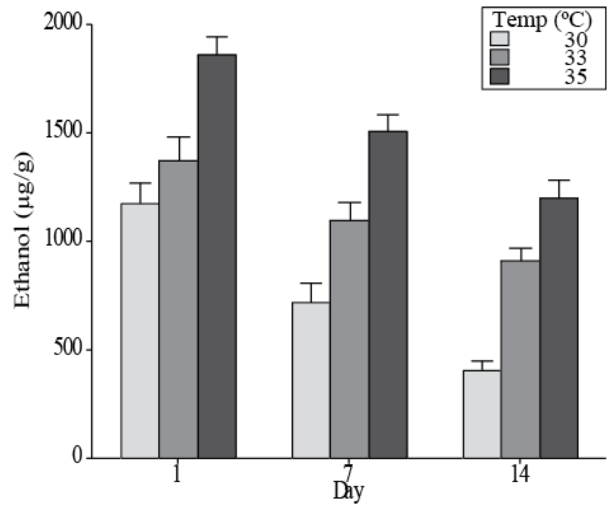

Fig.4. The effect of incubation temperature and storage period on ethanol of Kefir.

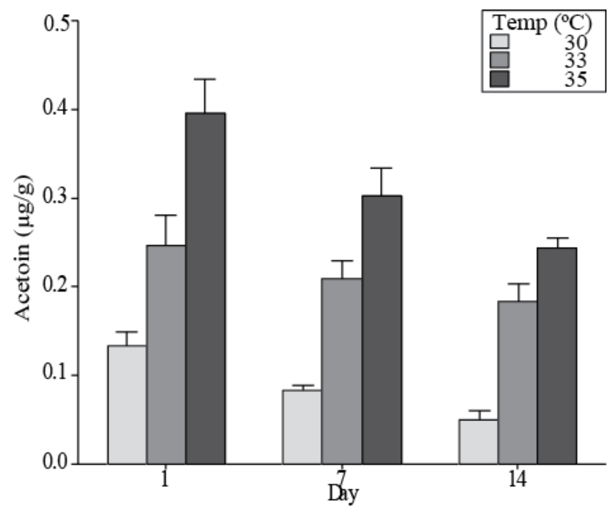

Fig.5. The effect of incubation temperature and storage period on acetoin of Kefir.

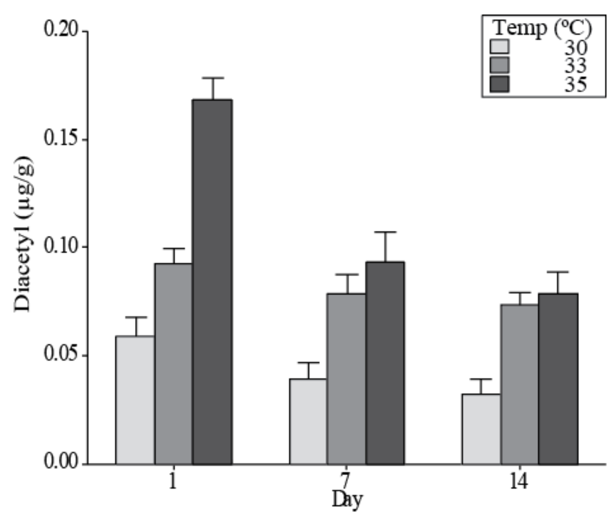

Fig.6.The effect of incubation temperature and storage period on diacetyl of Kefir.

Table 1 depicts the counts of the different microbial groups of kefir samples. Population of yeasts differed significantly ($p<0.05$). The number of yeasts increased significantly ($p<0.05$) with raising of temperature and decreased significantly ($p<0.05$) during cold storage. Yeasts counts on 7 day were similar to fresh product and decreased significantly ($p<0.05$) on 14 day. The results of this study were similar to findings of Liu and Lin (2000) for soymilk kefir. Number of lactococci differed significantly ($p<0.05$). Populations of lactococci increased significantly ($p<0.05$) with raising of temperature and decreased significantly ($p<0.05$) during cold storage Lactococci counts in fresh product (1 day) were nearly the same on 7 day but decreased significantly ($p<0.05$) on 14 day. Population of lactococci in this study was similar to results of Irigoyen *et al*. (2005) for milk kefir. Population of lactobacilli differed significantly ($p<0.01$). The number of lactobacilli increased significantly ($p<0.05$) with raising of temperature and decreased significantly ($p<0.05$) during cold storage. Lactobacilli counts on 7 day did not significantly differed from their counts on 1 day but decreased significantly ($p<0.05$) on 14 day. Irigoyen *et al*. (2005) reported similar results for lactobacilli counts in kefir. Population of lactobacilli was approximately similar to findings of Liu and Lin (2000) for soymilk kefir.

Table 1. Microbial counts of Kefir samples (values are means ± SD).

Milk Kefir	**Samples**	**Microbial**	**populations**	**(Log CFU/mL)**
T (ºC)	**Storage period (Days)**	**lactobacilli**	**lactococci**	**yeasts**
30	1	7.30±0.21	7.24±0.01	6.31±0.05
30	7	7.22±0.03	7.12±0.02	6.20±0.15
30	14	6.91±0.03	6.83±0.21	5.79±0.60
33	1	7.60±0.07	7.51±0.08	6.57±0.14
33	7	7.51±0.03	7.43±0.22	6.46±0.19
33	14	7.03±0.02	6.92±0.17	5.90±0.04
35	1	7.88±0.03	7.80±0.17	6.81±0.60
35	7	7.77±0.07	7.68±0.08	6.70±0.14
35	14	7.18±0.22	7.09±0.22	5.98±0.04

Table 2. Analysis of variance for physicochemical characteristics of kefir samples

Source	**df**	**MS**					
		Acetaldehyde	**Acetoin**	**Diacetyl**	**Ethanol**	**pH**	**Acidity**
Day	2	0.1819***	0.0228**	0.0051***	907641***	0.0059**	114.03**
Error 1 (Day)	6	0.0021	0.0012	0.0001	13348	0.0004	9.51
Temperature	2	0.1982***	0.1148***	0.0109***	1291307**	0.0065***	29.37***
Day × Temperature	4	0.0068*	0.0017**	0.0013***	18164*	0.0001	0.42
Error 2 (Residual)	12	0.0014	0.0002	0.0001	3644	0.0003	2.12

df: degree of freedom, MS: mean of squares; * $p<0.05$, ** $p<0.01$, *** $p<0.001$

Table 3 depicts the counts of the different microbial groups of kefir samples. Population of yeasts differed significantly ($p<0.05$). The number of yeasts increased significantly ($p<0.05$) with raising of temperature and decreased significantly ($p<0.05$) during cold storage. Yeasts counts on 7 day were similar to fresh product and decreased significantly ($p<0.05$) on 14 day. The results of this study were similar to findings of Liu and Lin (2000) for soymilk kefir. Number of lactococci differed significantly ($p<0.05$). Populations of lactococci increased significantly ($p<0.05$) with raising of temperature and decreased significantly ($p<0.05$) during cold storage Lactococci counts in fresh product (1 day) were nearly the same on 7 day but decreased significantly ($p<0.05$) on 14 day. Population of lactococci in this study was similar to results of Irigoyen et al. (2005) for milk kefir. Population of lactobacilli differed significantly ($p<0.01$). The number of lactobacilli increased significantly ($p<0.05$) with raising of temperature and decreased significantly ($p<0.05$) during cold storage. Lactobacilli counts on 7 day did not significantly differed from their counts on 1 day but decreased significantly ($p<0.05$) on 14 day. Irigoyen et al. (2005) reported similar results for lactobacilli counts in kefir. Population of lactobacilli was approximately similar to findings of Liu and Lin (2000) for soymilk kefir.

Table 3. Microbial counts of kefir samples (values are means ± SD)

Milk Kefir	Samples	Microbial	populations	(Log CFU/mL)
T (ºC)	**Storage period**	**lactobacilli**	**lactococci**	**yeasts**
30	1	7.30±0.21	7.24±0.01	6.31±0.05
30	7	7.22±0.03	7.12±0.02	6.20±0.15
30	14	6.91±0.03	6.83±0.21	5.79±0.60
33	1	7.60±0.07	7.51±0.08	6.57±0.14
33	7	7.51±0.03	7.43±0.22	6.46±0.19
33	14	7.03±0.02	6.92±0.17	5.90±0.04
35	1	7.88±0.03	7.80±0.17	6.81±0.60
35	7	7.77±0.07	7.68±0.08	6.70±0.14
35	14	7.18±0.22	7.09±0.22	5.98±0.04

Analysis of variance for microbial counts of kefir samples is shown in Table 4.

Table 4. Analysis of variance for microbial counts of kefir samples

Source	df	MS		
		lactobacilli	**lactococci**	**yeasts**
Day	2	1.8225^{***}	1.6300^{***}	1.4411^{***}
Error 1 (Day)	6	0.0340	0.0259	0.0342
Temperature	2	0.1654^{***}	1.5024^{***}	1.6825^{***}
Day × Temperature	4	0.0167^{***}	0.0089	0.0114
Error 2 (Residual)	12	0.0044	0.0077	0.0081

df: degree of freedom, MS: mean of squares; $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$

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