JOURNAL OF FOOD COMPOSITION AND ANALYSIS **13,** 35–43 (2000)
Article No. jfca.1999.0842
Available online at http://www.idealibrary.com on IDEAL®

ORIGINAL ARTICLE

Determination of Organic Acids and Volatile Flavor Substances in Kefir during Fermentation

Z. B. Güzel-Seydim*, A. C. Seydim†, A. K. Greene*,[1] and A. B. Bodine*

**Department of Animal and Veterinary Sciences, Clemson University, Clemson, SC 29634-0361, U.S.A and*
†Department of Food Science, Clemson University, Clemson, SC 29634-0371, U.S.A.

Received October 5, 1998, and in revised form September 14, 1999

The production of organic acids and volatile flavor components was measured during kefir starter culture fermentation. Samples were collected at 0, 5, 10, 15, and 22 h of fermentation (final pH = 4.6). Samples were analyzed for orotic, citric, pyruvic, uric, lactic, acetic, butyric, propionic and hippuric acids by HPLC. Acetaldehyde, ethanol, acetoin and diacetyl production were monitored using GC equipped with headspace autosampler. Levels of orotic, citric, and pyruvic acids slightly decreased during fermentation. Hippuric acid was totally consumed by 15 h of incubation. Acetic, propionic, and butyric acids and diacetyl were not detected. Production of ethanol began only after 5 h of incubation whereas acetaldehyde and acetoin increased during fermentation.

Key Words: kefir; organic acids; volatile flavor substances; fermentation; cultured dairy product.

INTRODUCTION

Kefir is a refreshing fermented milk beverage that has an exotic sour and slightly alcoholic flavor. Kefir is derived from "kef" which means pleasant taste in Turkish (Kurman *et al*., 1992). The product is made by inoculating milk with kefir grains. Kefir grains are small irregularly shaped, yellowish-white, hard granules which resemble miniature cauliflower blossoms. A good quality kefir has a foamy, pourable consistency. Kefir is a self-carbonated beverage that owes its distinctive flavor to a mixture of lactic acid, ethanol, carbon dioxide and other flavor products, such as acetaldehyde and acetoin. The unique flavor is the result of the symbiotic metabolic activity of a number of bacterial and yeast species (Vedamuthu, 1977). According to Koroleva (1988) "yeast exert a favorable effect on the activity of the lactic acid bacteria providing them with growth stimulants as well as by metabolizing some of the lactic acid".

In the preparation of traditional kefir, a starter culture must be produced from the kefir grains. Kefir grains are added to milk and incubated with stirring at 24–26°C until the pH is decreased to 4.6. After incubation, the kefir grains are removed via sieving and are reused in subsequent starter culture preparation. With each subsequent incubation, the size of the kefir grains increases slightly. The fresh kefir starter culture, sans grains, is added to pasteurized milk at the rate of 2–3% (v/v) and

[1]To whom correspondence and reprint requests should be addressed. Tel.: 864-656-3123. Fax: 864-656-3131. E-mail: agreene@clemson.edu

0889–1575/00/010035 + 09 $35.00/0

incubated at 24–26°C for approximately 18–20 h. After incubation, the product is refrigerated promptly.

Economically, fermented dairy products comprise an important segment of the dairy industry. Kefir is being investigated as a new product for wide-scale introduction into the U.S. market. The objective of this study was to determine the production of organic acids and volatile flavor substances through kefir fermentation.

MATERIALS AND METHODS

Materials

Kefir grains were provided by Dr Celalettin Kocak (Ankara University, Department of Dairy Science, Ankara, Turkey). Orotic, citric, pyruvic, lactic, uric, acetic, propionic, butyric and hippuric acids, acetaldehyde, acetoin and diacetyl for standards were purchased from Sigma Chemical Co., Inc. (St. Louis, MO).

Kefir Grain Maintenance

Upon receipt, kefir grains were inoculated into pasteurized milk or sterilized reconstituted non-fat dry milk and incubated at 25°C. Grains were transferred three times per week.

Kefir Sample Preparation

Kefir starter culture was prepared by inoculating 5 g kefir grains into 800 mL pasteurized whole milk. A 50 mL sample of milk was collected as the 0 h sample. Immediately after inoculation, milk was incubated at 25 ± 1°C on a rotary shaker (75–100 rpm). Samples were collected every 5 h during incubation. Sample pH was measured at the time of sampling, by using an ORION pH meter. The desired final pH of the product (4.5–4.6) occurred after approximately 22 h incubation.

Organic Acid Determination of Samples

A modification of the procedures of Marsili *et al.* (1981) and Fernandez-Garcia and McGregor (1994) was used for measuring the concentrations of organic acids in kefir samples. Four grams of each sample was diluted with 25 mL 0.01 N H_2SO_4, vortexed for 1 min, and refrigerated at 4°C for subsequent HPLC analysis. For the milk sample, the same procedure was used except that 0.013 N H_2SO_4 was used in place of 0.01 N H_2SO_4. Sulphuric acid-extracted samples were passed through 0.45 μm filters (Acrodisc™, Gelman Sciences, Ann Arbor, MI). Filtered samples were injected into a Rainin Rabbit Gradient HPLC system equipped with an Alltech IOA-1000 organic acid column (300 mm × 7.8 mm) maintained at 65°C. Degassed H_2SO_4 (0.009 N) was used as the mobile phase and the organic acids orotic, citric, pyruvic, lactic, uric, acetic, propionic, butyric and hippuric were detected using UV detection at 275 nm. A 10 μL injection volume was used for both samples and standards. Organic acids were quantified using external standards. Standard organic acid solutions were prepared in distilled deionized water (ddH_2O) filtered through 0.45 μm Gelman Acrodisc™ filters (Ann Arbor, MI). HPLC chromatograms and quantification were obtained using Rainin Dynamax Method Manager software. Standard curves based

on peak height were calculated for the individual organic acids covering a broad range of concentrations.

Determination of Volatile Flavor Substances

For the volatile component analysis, 5 mL kefir starter culture was transferred into headspace vials (20 mL). The vials were heated at 85°C for 5 min using a headspace autosampler (Model 7000, Tekmar Corp., Cincinnati, OH). Headspace samples were automatically injected onto a 30 m fused silica capillary column (DB-5, J&W Scientific, Folsom, CA) (0.32 i.d. × 30 × 1 μm) maintained at 40°C. The column was temperature programmed from − 20 to 30°C at 5°C/min and 30 to 220°C at 10°C/min. Helium (flow of 30 mL/min) was used as the carrier gas. The flame ionization detector signals were stored and integrated using computer software (HP 3365 Series II Chemstation, Hewlett Packard Corp., Wilmington, DE). Peak identification of volatile substances was based on the retention times of the individual reference standards. Peak identification was also verified by a headspace sampler (HP 7694) integrated to a gas chromatograph (HP 6890, GC System) equipped with a mass-selective detector (HP 5973) running on total ion mode. The same headspace conditions were used to maintain the consistency of the two detectors. Using the headspace conditions as described previously, sample headspace was automatically injected onto an HP5-MS 95% dimethyl siloxane copolymer column (30 m × 250 μm × 0.25 μm) (Hewlett-Packard Corp., Wilmington, DE) with a flow of 1.5 mL/min. Peak integration was performed on a personal computer using HP Vectra X_M software. Spectra were matched with the HP-MS ChemStation® Libraries, including Wiley Library, NIST Library of Mass Spectra and Subsets, (Hewlett-Packard Corp., Wilmington, DE). The GC/MS analysis was used for verification of peak identification only. Standard solutions of acetaldehyde, acetoin, diacetyl and ethanol were prepared with distilled deionized water. Standard solutions were analyzed as described previously for the samples. Quantification of the volatile components in the experimental samples was accomplished from the external standard curves of these individual substances.

Statistical Analysis

The data were analyzed using one-way analysis of variance (ANOVA) by SAS® (SAS Institute, Inc., 1990). 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 ($\alpha = 0.05$). Kefir samples were prepared on five different days. All samples were analyzed in duplicate.

RESULTS AND DISCUSSION

The bacteria most frequently isolated from kefir are homofermentative and heterofermentative *Lactobacillus*, *Lactococcus*, *Leuconostoc* and some *Acetobacter* (Ergüllü and Ucuncu, 1983, Duitschaever *et al*., 1988; Koroleva, 1988; Angulo *et al*., 1993; Rea *et al*., 1996). Many researchers have studied the composition of the microflora present in kefir grains. They have isolated *Streptococcus lactis* (Kandler and Kunath, 1983), *Lactobacillus brevis* (Marshall *et al*., 1984; Angulo *et al*., 1993), *Lactobacillus viridescens*, *Lactobacillus gasseri*, *Lactobacillus fermentum* and *Lactobacillus casei* (Angulo *et al*., 1993), *Lactobacillus kefir* (Kandler and Kunath, 1983; Marshall *et al*., 1984; Angulo *et al*., 1993), *Lactobacillus acidophilus* (Angulo *et al*., 1993; Marshall, 1993), *Leuconostoc* (Rosi and Rossi, 1978), *Lactobacillus kefiranofaciens* (Toba *et al*., 1987; Mukai

et al., 1992), *Lactobacillus kefirgranum* and *Lactobacillus parakefir* (Takizawa *et al*., 1994) from kefir grains. Both the homofermentative or heterofermentative pathways are employed by these bacteria, leading to an acidified product.

The process of acidification is reported in Figure 1, showing a drop in pH values over a 22 h fermentation period. On average, the pH of the whole milk prior to inoculation with kefir grains was 6.7. The pH progressively decreased until completion of fermentation. There was no significant difference between pH at 0, 5, and 10 h ($P > 0.05$); however, a significant difference was detected between pH in the first 10 h versus after 15 h ($P < 0.05$). Organic acids may occur in dairy products as a result of hydrolysis of butterfat (fatty acids), biochemical metabolic processes, or bacterial metabolism. Lactate is a common end product of bacterial fermentation. The measured concentrations of lactate (Fig. 2) indicated that lactate production slowly increased during the first 10 h of fermentation, then rapidly and significantly increased ($P < 0.05$) between 10 and 15 h of fermentation from 590 to 3700 μg/g.

Lactose is readily degraded to galactose and glucose by Group N streptococci. In the homofermentative pathway, glucose is further metabolized by the Embden–Meyerhoff–Parnas (EMP) pathway to pyruvate. Pyruvate is used directly as an H-acceptor, and 2 mol of lactate are formed per glucose molecule. However, the heterofermentative pathway yields theoretically 1 mol each of lactate, ethanol and CO_2. In plain yogurt, which is produced by lactic acid fermentation, lactic acid content was reported as 8760 μg/g (Fernandez-Garcia and McGregor, 1994) and 14550 μg/g (Marsili *et al*., 1981). In this study, kefir cultures produced 6400 μg/g lactic acid upon completion of fermentation. Thus, kefir has a lower lactic acid content than yogurt. This may be due to the preferential use of a heterofermentative pathway with a resultant production of CO_2. A common problem in yogurt production is overacidification. Since kefir does not have a high acid production, overacidification problem is not likely to occur.

Formation of pyruvate was detected after 5 h kefir fermentation (Fig. 2). Pyruvate content increased slightly during fermentation, concurrent with lactate production between 0 and 10 h and between 15 and 22 h. A significant increase in pyruvate levels occurred during 15–22 h of fermentation ($P < 0.05$). *S. lactis*, a common component of the kefir culture, uses the EMP pathway to produce pyruvate. Pyruvate is preferen-

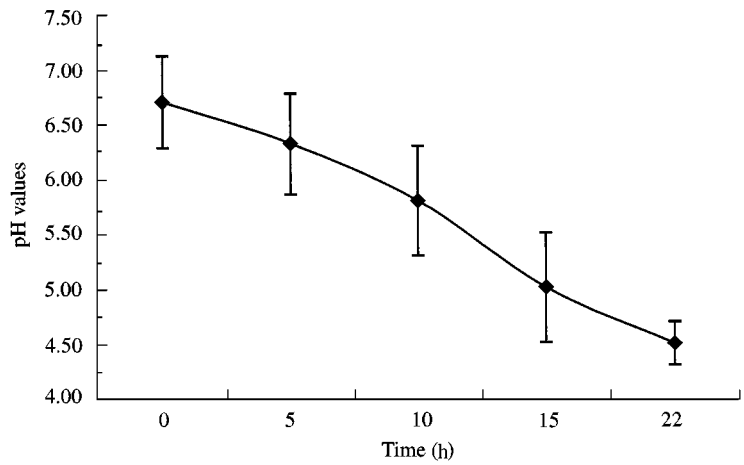

FIGURE 1. Change in pH during kefir fermentation.

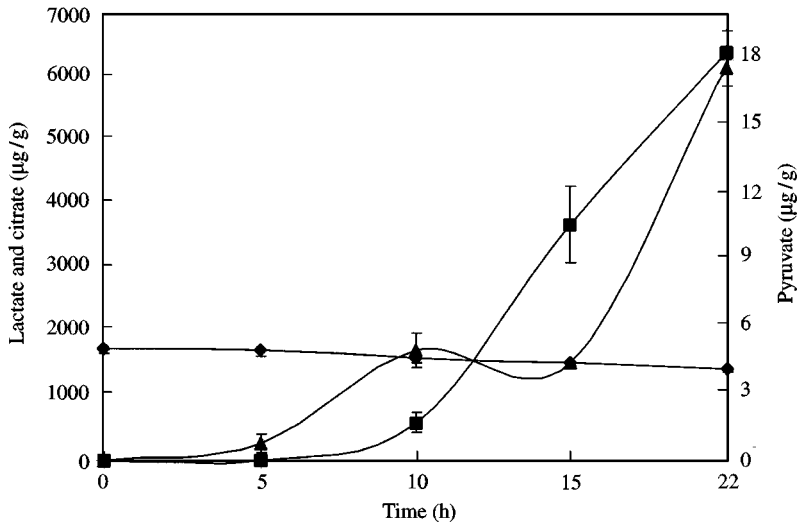

FIGURE 2. Concentration of citrate, lactate and pyruvate during kefir fermentation: —◆—, citrate; —■—, lactate; —▲—, pyruvate.

tially converted to lactate and residual pyruvate is converted to acetaldehyde and diacetyl. Similarly, the concentration of pyruvate in cultured buttermilk has been reported to increase during fermentation (Marsili, 1981).

The mean citrate concentration of the milk control (0 h) was 1760 μg/g citrate (Fig. 2). After 15 h of fermentation, the amount of citrate had decreased to 1600 μg/g. Upon completion of fermentation, the citrate level had decreased to 1440 μg/g although there was no statistically significant difference in citrate concentration between any of the incubation periods ($P > 0.05$). Citrate is the preferred substrate for acetoin and diacetyl formation by some lactic acid bacteria.

Orotic acid concentration decreased significantly ($P < 0.05$) after 15 h fermentation (Fig. 3). Orotic acid is an intermediate product generally involved in the synthesis of nucleotides which may explain its decline (Gottschalk, 1986). Orotate is a free pyrimidine and forms orotidylate in the presence of orotate phosphoribosyl transferase.

Hippuric and uric acids also decreased during fermentation (Fig. 3). After 5 h, hippuric acid concentrations significantly decreased ($P < 0.05$) and could not be detected by the end of fermentation. Similar decreases in hippuric acid content have been reported during yogurt fermentation (Fernandez-Garcia and McGregor, 1994). Nishimoto *et al.* (1969) reported that hippuric acid is a precursor in the synthesis of benzoic acid by lactic acid bacteria. Therefore, it is possible that hippuric acid disappeared as a consequence of growth of lactic acid bacteria, particularly in the last 10 h of fermentation. After 15 h, uric acid concentrations significantly decreased ($P < 0.05$).

Acetic, propionic and butyric acids were not detected during kefir fermentation. However, Rea *et al.* (1996) reported acetate formation in kefir from samples obtained from various locations throughout Ireland. This result might be due to the variations in the ratio and types of micro-organisms in kefir grains. The source and growth conditions of kefir grains are thus of great importance in kefir flavor.

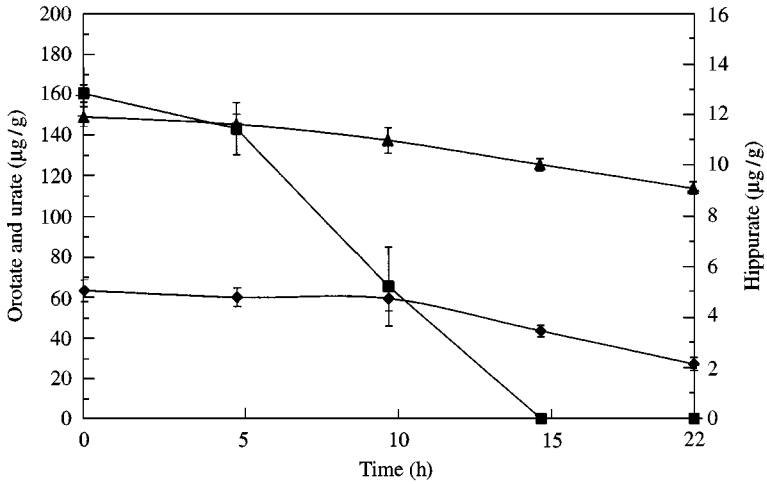

FIGURE 3. Concentration of urate, orotate and hippurate during kefir fermentation: —◆—, urate; —▲—, orotate; —■—, hippurate.

Acetaldehyde, diacetyl and acetoin are important flavor substances for many types of cultured dairy products. A typical chromatogram of the results of the head space analysis indicated the presence of many compounds in fermented kefir (Fig. 4). Only four compounds, namely acetaldehyde, acetoin, diacetyl and ethanol were sought in this study. Changes in production of acetaldehyde, acetoin and ethanol during kefir fermentation are shown in Figure 5. In this study, acetaldehyde was first detected at 10 h and its concentration continued to increase significantly ($P < 0.05$) after 15 h. The average final concentration of acetaldehyde was only 5 µg/g, whereas plain yogurt is reported to have an acetaldehyde content ranging from 23 to 41 µg/g (Gorner *et al*., 1973). Acetaldehyde can be converted to ethanol by alcohol dehydrogenase (Tamime and Robinson, 1983). This may explain the lower amount of acetaldehyde observed in kefir samples.

Yeasts are primarily responsible for the alcohol production in kefir. The yeasts commonly isolated from kefir grains are *Saccharomyces cerevisiae* (Rohm *et al*., 1992; Angulo *et al*., 1993), *Candida kefir* (Angulo *et al*., 1993), the imperfect form of *Kluyveromyces lactis* (Engel *et al*., 1986; Angulo *et al*., 1993; Marshall, 1993), *Saccharomyces delbruecki* (Engel *et al*., 1986), *Torulopsis holmii* (Iwasawa *et al*., 1982), *Candida holmii* and *Saccharomyces unisporus* (Engel *et al*., 1986; Angulo *et al*., 1993), *Kluyveromyces marxianus* (Rohm *et al*., 1992), *Torulospora delbrueckii* and *Candida friedricchi* (Angulo *et al*., 1993). Although yeasts are commonly recognized for ethanol-producing ability, the bacterium, *L. kefir*, is a heterofermentative lactobacillus capable of producing ethanol (up to 0.25%) and CO_2 (Marshall, 1984). Ethanol production during kefir fermentation is presented in Figure 5. Only slight ethanol production was noted at approximately 5 h of fermentation; a significant increase ($P < 0.05$) was recorded by 15 h of fermentation with a final ethanol concentration of 0.04% (v/v) occurring by 22 h. There are notable variations among the reported ethanol contents of kefir (0.01–1.0%) (Kurman *et al*., 1992; Marshall and Cole, 1985; Vedamuthu, 1977). Higher alcohol content may be associated with a yeastly flavor and, in fact, authentic kefir does have a very slight yeasty flavor (Kroger, 1993; Vedamuthu, 1977).

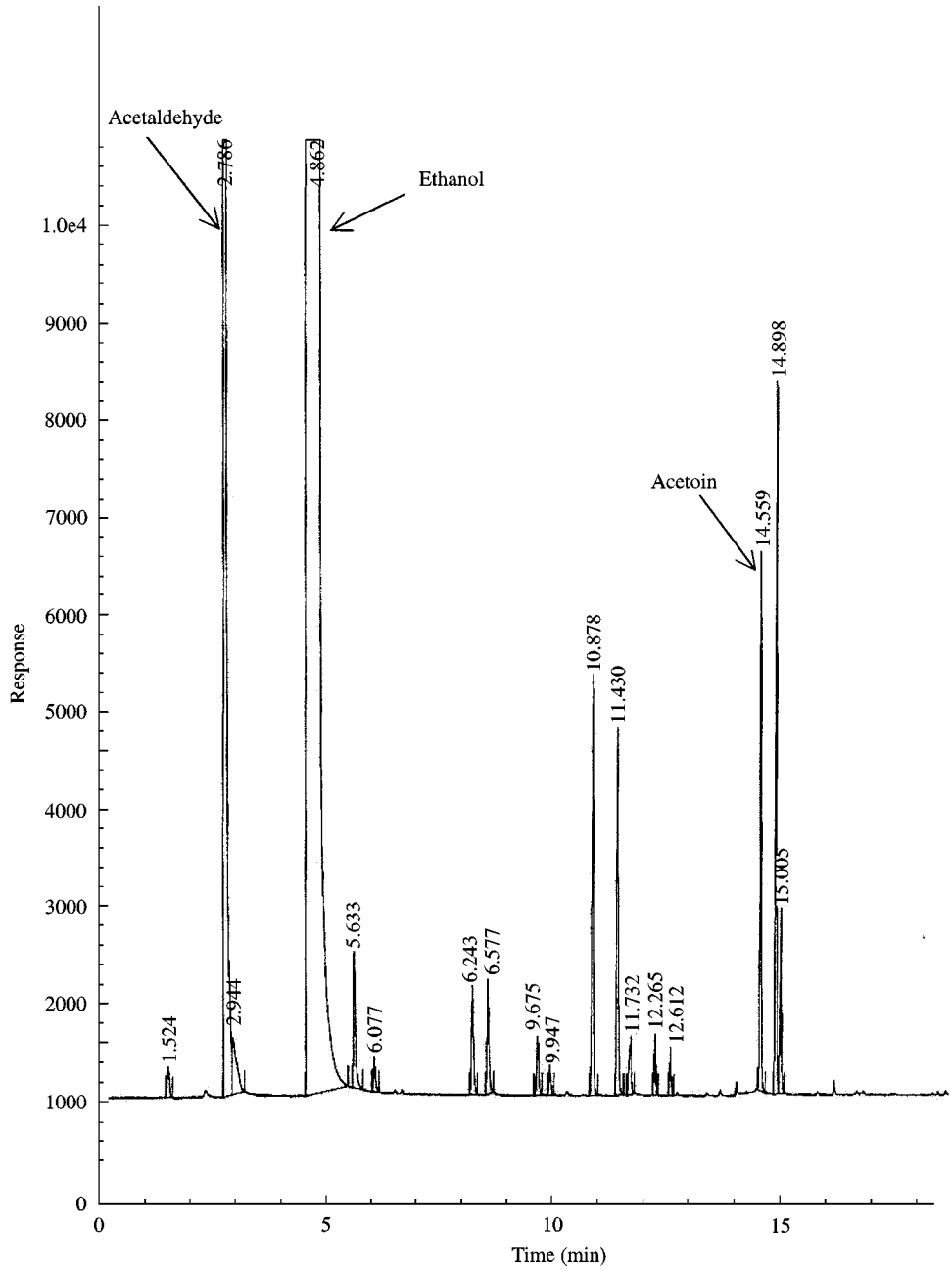

FIGURE 4. Typical chromatogram of head space analysis of fermented kefir.

In this study, acetoin was significantly increased ($P < 0.05$) by 10 h of fermentation to a maximum of 25 μg/g by 15 h (Fig. 5). Chuang and Collins (1968) reported that production of acetoin by yeasts is stimulated by acetaldehyde addition. *L. brevis* is a heterofermentative lactic acid bacterium that may produce CO_2, acetate and lactate

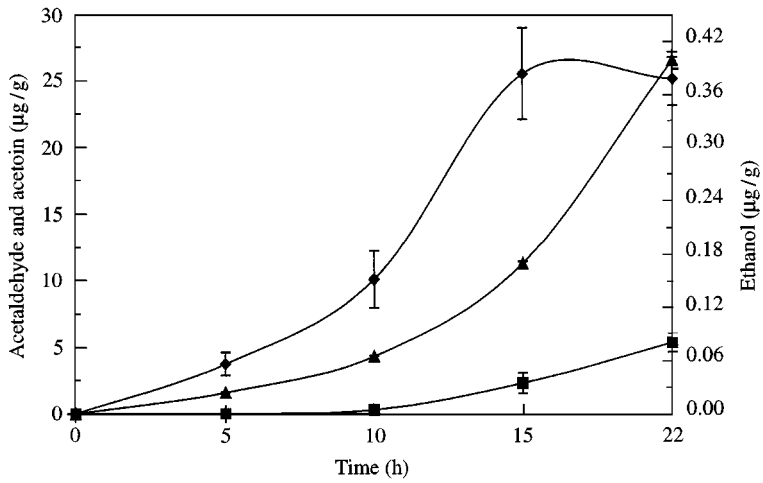

FIGURE 5. Concentration of acetoin, acetaldehyde and ethanol during kefir fermentation: —◆—, acetoin; —▲—, acetaldehyde; —■—, ethanol.

from glucose metabolism via pyruvate and, since it is able to use citrate, it may also produce acetoin and diacetyl. In this study, diacetyl was not detected using the described experimental procedure.

In this study, urate, orotate and hippurate all decreased during kefir starter culture fermentation. Lactate, pyruvate, acetaldehyde, acetoin and ethanol all increased during fermentation.

CONCLUSION

Kefir develops a unique flavor profile during fermentation. It contains amounts of flavor components different than those noted in yogurt and other known cultured dairy products. In this experiment, no diacetyl was detected; diacetyl is a common flavor component of most cultured dairy products. As typical of other cultured dairy products, the pH of kefir samples progressively decreased until completion of fermentation.

ACKNOWLEDGEMENTS

The authors wish to thank Ms Rebecca S. Rodgers, Dr Inyee Y. Han and Dr Paul L. Dawson for technical assistance, Dr Celalettin Kocak for kindly providing kefir grains and Gaziosmanpasa University and Clemson University for support. The authors also wish to thank Dr M. J. B. Paynter for reviewing this manuscript.

REFERENCES

Angulo, L., Lopez, E., and Lema, C. (1993). Microflora present in kefir grains of the Galician region (North–west of Spain). *J. Dairy Res.* **60,** 263–267.

Chuang, L. F., and Collins, E. B. (1968). Biosynthesis of diacetyl in bacteria and yeast. *J. Bacteriol.* **95,** 2083–2087.

Duitschaever, C. L., Kemp, N., and Smith, A. K. (1988). Microscopic studies of the microflora of kefir grains and kefir made by different methods. *Milchwissenschaft* **43,** 479–481.

Engel, G., Krusch, U., and Teuber, M. (1986). Microbiological composition of kefir. I. Yeasts. *Milchwissenschaft*. **41,** 418–421.

Ergüllü, E., and Üçüncü, M. (1983). Kefir microflorasi uzerinde arastirma. *Gida* **8,** 3–10.

Fernandez-Garcia, E., and McGregor, J. U. (1994). Determination of organic acids during the fermentation and cold storage of yogurt. *J. Dairy Sci.* **77,** 2934–2939.

Fujisawa, T., Benno, Y., Yaeshima, T., and Mitsuoka, T. (1992). Taxonomic study of the *Lactobacillus gallinarum* sp. nov. and *Lactobacillus johnsonii* sp. nov. and synonymy of *Lactobacillus acidophilus* group A3 with the type strain of *Lactobacillus amylovorus*. Int. J. Syst. Bacteriol. **42,** 565–568.

Gorner, F., Palo, V., and Seginnova, M. (1973). Aroma compounds in cultured milks, *Dairy Sci. Abstract* **35,** 317.

Gottschalk, G. (1986). *Bacterial Metabolism*, 2nd edn. Springer-Verlag, New York.

Iwasawa, S., Ueda, M., Miyata, N., Hirota, T., and Ahiko, K. (1982). Identification and fermentation character of kefir yeast. *Agric. Biol. Chem.* **46,** 2631–2636.

Kandler, O., and Kunath, P. (1983). Lactobacillus kefir sp. nov., a component of the microflora of kefir. *Syst. Appl. Microbiol.* **4,** 286–294.

Koroleva, N. S. (1988). Starters for fermented milks. Section 4: kefir and kumys starter. *Bulletin of the IDF* 227, Chap. 2. International Dairy Federation, Square Vergotte, Brussels, Belgium.

Kurman, J. A., Rasic, J. L., and Kroger, M. (1992). *Encyclopedia of Fermented Fresh Milk Products*. Van Nostrand Reinhold, New York.

Marshall, V. M. (1993). Starter cultures for milk fermentation and their characteristics. *J. Soc. Dairy Technol.* **46,** 49–56.

Marshall, V. M., and Cole, W. M. (1985). Methods for making kefir and fermented milks based on kefir. *J. Dairy Res.* **52,** 451–456.

Marshall, V. M., Cole, W. M., and Brooker, B. E. (1984). Observations on the structure of kefir grains and the distribution of the microflora. *J. Appl. Bacteriol.* **57,** 491–497.

Marsili, R. T., Ostapenko, H., Simmons, R. E., and Green, D. E. (1981). High performance liquid chromatographic determination of organic acids in dairy products. *J. Food. Sci.* **46,** 52–57.

Mukai, T., Onose, Y., Toba, T., and Itoh, T. (1992). Presence of glycerol teichoic acid in the cell wall of *Lactobacillus kefiranofaciens*. *Lett. Appl. Microbiol.* **15,** 29–31.

Nishimoto, T., Uyeta, M., and Tane, S. (1969). Precursor of benzoic acid in fermented milk. *J. Food Hyg. Soc. Jpn.* **10,** 410.

Rea, M. C., Lennartsson, T., Dilon, P., Drinan, F. D., Reville, W. J., Heapes, M., and Cogan, T. M. (1996). Irish kefir-like grains: their structure, microbial composition and fermentation kinetics. *J. App. Bacteriol.* **81,** 83–94.

Rohm, H., Eliskases-Lechner, F., and Braver, M. (1992). Diversity of yeasts in selected dairy products. *J. Appl. Bacteriol.* **72,** 370–376.

Rosi, J., and Rossi, J. (1978). The kefir microorganisms: the lactic acid bacteria. *Sci. Technol. Lattiero Casearid.* **29,** 291–305.

SAS Institute, Inc. (1990). *SAS/STAT® User's Guide*, Version 6, 4th edn. V2 GLM-VARCOMP, Cary, NC.

Takizawa, S., Kojima, S., Tamura, S., Fujinaga, S., Benno, Y., and Nakase, T. (1994). *Lactobacillus kefirgranum* sp. nov. and *Lactobacillus parakefir* sp. nov., two new species from kefir grains. *Int. J. Syst. Bacteriol.* **44,** 435–439.

Tamime, A. Y., and Robinson, R. K. (1983). *Yogurt Science and Technology*. Pergamon Press, Oxford, U.K.

Toba, T., Arihara, K. and Adachi, S. (1987). Comparative study of polysaccharides from kefir grains, an encapsulated homofermentative *Lactobacillus* species and *Lactobacillus kefir*. *Milchwissenschaft*. **42,** 565–568.

Vedamuthu, E. R. (1977). Exotic fermented dairy foods. *J. Food Prot*. **40,** 801–802.