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Production of Hard-Type Cheese Using Free or Immobilized Freeze-Dried Kefir Cells as a Starter Culture

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This study provides a contribution to hard-type cheese starter culture production through the use of a freeze-dried culture in the ripening of hard-type cheeses. The effect of initial cell concentration, ripening temperature, and cell immobilization of kefir on the degree of openness, mold spoilage, microbial associations, physicochemical characteristics, and aroma-related compounds was studied. Use of kefir starter cultures resulted in cheese with an increased shelf life and resistance to spoilage as compared to control cheeses without kefir inoculants. Furthermore, the freeze-dried kefir culture improved aroma, taste, and texture characteristics while increasing the degree of openness in comparison to traditional hard-type cheese products. The kefir culture resulted in an increase in counts of total aerobic bacteria, yeasts and molds, lactococci, and lactobacilli until the 15th day of ripening. From then on, only lactobacilli counts increased, reaching levels up to 9.17 log CFU/g in cheeses ripened at 5 °C using freeze-dried kefir cells immobilized on casein. SPME-GC/MS analysis revealed major differences in volatile composition, especially with regard to alcohols (up to 75%), carbonyl compounds (up to 75%), and esters (up to 64%) between cheeses made with kefir cells and cheeses made without kefir inoculants.

KEYWORDS: Kefir; hard-type cheese; volatiles; ripening; openness

INTRODUCTION

It is generally accepted that the preference for hard-type cheese products is attributed to the full creamy flavor that along with the salty taste and unique sensory characteristics offer this type of cheese a distinct character. Hard-type cheeses are made from raw or pasteurized milk from ewes, goats, cows, buffalo, or a mixture of these. Quite early in the production process, cheese products are pressed and then ripened for at least 3 months while being frequently subjected to salting and rubbing, especially during the first period of ripening. Hard-type cheeses are characterized by low moisture content, high fat content, and a salty flavor. Variations in production process and/or limitations in the origin of milk have led to the production of hard-type cheeses such as Graviera Kritis, Kefalograviera, Cheddar, Gouda, Parmesan, Parmigiano-Reggiano, and Gruyère de Comté.

The main focus of research on cheese production in the last two decades has been on the improvement of quality characteristics and to the production of healthier cheese. While key objectives of the latter mainly constitute the reduction of fat, salt (*1, 2*), and the addition of probiotic properties to the final product (*3–6*), improvement of quality characteristics involves efforts on the improvement of texture, degree of openness or eye formation, flavor, and taste of the dairy product (*7–9*).

To this end, use of an appropriate starter culture has gained a substantial role in cheese manufacturing. The main role of starter cultures is still the conversion of the primary sugar in milk lactose to lactic acid, which is substantial for the safe preservation of the product. However, the biochemical processes of glycolysis, lipolysis, and proteolyzis, which lead to the formation of key components of flavor and aroma, are directly and indirectly dependent on the microbial associations of starter and nonstarter cultures during cheese manufacturing (*10, 11*). Evaluation of both individual and mixture of strains as starters in cheese production has demonstrated the applicability of a variety of cultures of bacteria such as bifidobacteria (*12*), *Lactococcus* (*13–16*), *Lactobacillus* (*9, 13*), *Leuconostoc*, and *Enterococcus* species (*13*).

Recently, the kefir culture has gained researchers' attention with regard to cheese manufacturing due to its potential effect on quality, health, and safety properties of the final product. Kefir is a consortium of microbes that is mainly used in the production of the low alcohol traditional Russian drink, kefir, where milk constitutes the initial fermenting substrate. This

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multistrain culture has been shown to include various yeasts, lactococci, and acetic acid bacteria along with strains of *Lactobacillus* (*Lactobacillus acidophilus*, *Lactobacillus casei*, and *Lactobacillus reuteri*) that are considered to have probiotic properties (*17*). Upon evaluation in white soft cheese production, the kefir culture revealed its ability to suppress potent spoilage and pathogenic microorganisms during ripening while offering a rich aromatic character to the final product by increasing the variety of esters, free fatty acids, alcohols, and carbonyl compounds (*18*). This culture was subsequently tested in the dried form as a starter in whey-cheese production (*19*) and was immobilized on apple pieces in whey fermentation (*20*), establishing its potential to produce a plethora of aromatic compounds in lactose-rich substrates. More recently, the kefir culture immobilized on casein was characterized by increased stability. Indeed, casein appeared to constitute a valuable support for kefir culture immobilization, as this new biocatalyst (dried or wet) retained its fermentation activity for up to 2 months of repeated whey fermentations while, in the dried form, showed a high cell viability even after 30 days of storage at 4−6 °C (*21*). It is well-demonstrated that drying constitutes the method of choice for long-term preservation of microorganisms and biocatalysts, as it retains their stability and is more convenient for commercial needs regarding transportation, storage, and cell dosage. Despite the variety of drying methods that have been tested or used, it has been demonstrated that there is no generic method for all applications (*22*). However, freeze-drying still remains the most popular method in the microbiological industry mainly due to the convenience it offers in shipping and handling of aliquots of microorganisms (*22*).

The kefir culture or similar multistrain cultures have not been tested yet in hard-type cheese production where long ripening periods are necessary for the product to acquire its organoleptic character. The aim of this work was to study the impact of freeze-dried kefir as a new starter culture in the production of hard-type cheese. Data revealing the effect of kefir culture on aroma, flavor, textural characteristics, degree of openness, and microbial associations throughout the ripening process are presented and compared with cheeses made with kefir immobilized on casein. Changes in volatile profiles associated with the use of a freeze-dried kefir culture are presented and discussed.

MATERIALS AND METHODS

Production of Freeze-Dried Kefir Culture. A multistrain culture, kefir, isolated from a commercial kefir drink, was used as the starter culture in cheese production in this study. Kefir was routinely grown at 30 °C on a sterilized (130 °C for 15 min) synthetic medium consisting of 4% lactose, 0.4% yeast extract, 0.1% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, and 0.5% $MgSO_4 \cdot 7H_2O$ (Merck, Darmstadt, Germany). Cells were harvested by centrifugation at 5000 rpm for 10 min. Amounts containing approximately 1.0 g of cells were resuspended in cheese whey (~50 mL) and incubated at 30 °C for ~24 h for further production of kefir culture when needed. The freeze-dried kefir culture was obtained by resuspending 5.0 g of pressed wet cells in 5 mL of fermented whey, used as a cryoprotecting agent, and then frozen to −45 °C. The frozen samples were freeze-dried overnight at 5×10^{-3} bar and −45 °C in a freeze-drying system (Freezone 4.5, Labconco, Kansas City, MO).

Production of Freeze-Dried Kefir Immobilized on Casein. Casein was isolated from commercial pasteurized bovine milk (0% fat) as described previously (*21*). Immobilization of kefir cells on casein was performed by the first method described by Dimitrellou et al. (*21*) followed by freeze-drying as described in the same study.

Cheese Manufacture and Sampling. Bulk ewes' milk was obtained from a local dairy factory (A.VI.GAL. S.A., Elliniko, Farres, Achaia) and was standardized, by separation, to 6.0% fat followed by pasteurization at 72 °C for 15 s and cooling to 33 °C. If not otherwise stated, free or immobilized cells of freeze-dried kefir culture were added to the milk, and after agitation, the liquid mixture was left at 33 °C for 30 min. Subsequently, commercial cheese rennet (Vlacha, Vlachopoula Ltd., Athens, Greece) was added at a concentration of 0.01% w/w, and the mixture was left undisturbed at 33 °C for 25 min for curd formation. The curd was cut into 2.5−3.0 cm cubes, left to rest for 2−3 min, and further cut into kernel-sized pieces. It was then stirred very gently and slowly for 20 min. Curd particles and whey were gradually scalded from 33 to 48 °C over a period of 25 min with continuous stirring. Stirring was continued at 48 °C for 20 min to obtain curd particles of the desired firmness. The curd pieces were wrapped in a cheesecloth and placed into molds and then pressed at 0.22 kg/cm^2 for 20 min. Afterward, the cheese was removed from the mold, the cheesecloth was changed, and the cheese was inverted and pressed again. After 60 min, the cheese was similarly overturned and pressed for another 60 min. When pressing was completed, the cheesecloth was removed, and the cheese was left in the mold overnight at 14−16 °C. The finished cheese product weighed 550−680 g, and its dimensions were ~14 cm in diameter and 5 cm high. The next day, after removal of the molds, the cheese was salted in brine of 19 °Be at 14−16 °C for 24 h. At 20 °C the relationship between specific gravity (s. g.) and degrees Baumé (°Be) is for liquids heavier than water: s. g. = 145/(145 − degrees Baumé) while for liquids lighter than water: s. g. = 140/(degrees Baumé + 130). It was then transferred to a clean board where it remained for 10 days at 14−16 °C. During this time, the cheese was inverted every second day, wiped with a dry cheesecloth, and rubbed with dry salt. Cheese ripening was completed at 5 °C, 18 °C, or room temperature (20−22 °C).

Cheeses were designated as follows: cheese A or rennet cheese (hard-type cheese without any starter culture), cheese B (hard-type cheese produced using as an inoculum of 0.5 g of kefir culture/L of milk), cheese C (hard-type cheese produced using 1.0 g of kefir culture/L of milk as an inoculum), cheese D (hard-type cheese produced using 2.0 g of kefir culture/L of milk as an inoculum), and cheese E (hard-type cheese produced using 1.0 g of kefir culture immobilized on casein/L of milk as an inoculum). Rennet was added in all cheese samples. All experiments were repeated 3 times. Ripening of the produced cheeses was monitored at 5 °C, 18 °C, and room temperature for 90 days. Samples from each cheese were taken after 0, 15, 30, 60, and 90 days of ripening.

Chemical Analysis. Samples for chemical analysis were prepared by 20 g of cheese macerated with warm water (40 °C) up to a total volume of 210 mL followed by filtration. Filtrates were assayed for lactic acid, ethanol, residual sugar, and cheese samples for moisture content (*23*). Acidity was determined by titration with 0.1 N NaOH using phenolphthalein as the indicator (*24*) and expressed as lactic acid content. Residual sugar (lactose, glucose, and galactose) was determined by HPLC, and ethanol was determined by GC, as described by Kopsahelis et al. (*20*). Cheese pH was determined according to the British standard method of pH (BS 770: Part 8).

SPME-GC/MS) Analysis. Cheese samples ripened at 5 and 18 °C for 90 days were assayed for volatile compounds using SPME-GC/MS analysis. Grated cheese samples (7 g each) were placed in a 20 mL headspace vial fitted with a Teflon-lined septum sealed with an aluminum crimp seal, through which the SPME syringe needle (Supelco, Bellefonte, PA) was introduced. The internal standard was methyl octanoate (Merck, Darmstadt, Germany) at a final concentration of 125.29 μg/kg cheese. The sample was then incubated at 80 °C for 30−35 min (*25*), and the absorbed volatile compounds were then determined by GC/MS. GC/MS analysis was performed on a Shimadzu GC-17A coupled to a GCMS-QP5050A mass spectrometer. A Supelcowax-10 column (60 m, 0.32 mm i.d., and 0.25 μm film thickness) was used. Helium was used as the carrier gas (linear velocity of 1.5 mL/min). The oven temperature was set as follows: 35 °C for 3 min, a temperature gradient of 5 °C/min up to 110 °C, a temperature gradient of 10 °C/min up to 240 °C, and a final extension at 240 °C for 10 min. The injector was operated in splitless mode at 280 °C, the detector temperature was set at 250 °C, and the mass spectrometer was operated in electron impact mode with the electron energy set at 70 eV. Identification was achieved by comparison to standard compounds and

Figure 1. Cross-sections of hard-type cheeses after 15 days of ripening, demonstrating the effect of initial freeze-dried kefir starter culture on texture and gas hole formation: (**A**) rennet cheese, (**B**) 0.5 g kefir/L milk, (**C**) 1.0 g kefir/L milk, and (**D**) 2.0 g kefir/L milk.

data obtained from NIST107, NIST21, and SZTERP libraries as described recently by Kopsahelis et al. (*20*). The volatile compounds were determined by dividing the peak areas of the compounds of interest by the peak area of the internal standard and multiplying this ratio by the initial concentration of the internal standard. Peak areas were calculated from the full scan chromatograph by using the total ion current (TIC).

Microbiological Analysis. The inoculum for microbiological analysis was obtained by blending 10 g of each cheese sample with 90 mL of sterilized Ringer solution (1/4 strength) followed by serial dilutions. Cheese samples were obtained from the cheese interior. Microbiological assays involved the determination of total aerobic counts on a plate count agar (Fluka 70188, Sigma-Aldrich, Athens, Greece) at 30 °C for 120 h, enumeration of yeasts and molds after incubation on potato glucose agar (Fluka 70139) at 30 °C for 120 h, enumeration of lactococci (Gram positive, catalase negative) after incubation on M-17 agar (Fluka 63016) at 37 °C for 120 h, and enumeration of lactobacilli (Gram positive, catalase negative) after incubation on acidified MRS agar (Fluka 69964) at 37 °C for 120 h. Gram staining and catalase tests were performed for the confirmation of lactic acid bacteria. Results are presented as the log mean of CFU on culture plates containing between 30 and 300 colonies per gram of cheese. Microbiological analysis was performed in duplicate using duplicate cheese samples.

Sensory Evaluation. Sensory assessments were made by an internal panel consisting of 10 previously trained members. Cheese samples ripened for 90 days were cut into pieces (2.5 cm × 2.5 cm × 2.5 cm), placed on gray plain plates, and left for 1 h to reach room temperature (~20 °C). The coded samples were served to tasters in individual booths with natural daylight, in a randomized order (*26*). The panel was asked to score cheeses for taste, aroma, degree of openness, and textural characteristics on a scale of 1−7 (1, unacceptable; 3, objectionable; 5, good; and 7, superior) (*27*). Degree of openness and formation of molds were monitored by visual inspections of cheeses, and their cross-sections were photographed using a Canon A430 camera (Canon Inc., Hong Kong). Kefir cheese samples were compared to commercial hard-type cheeses such as Cheddar (U.S.), Emmental (Switzerland), Parmigiano-Reggiano (Italy), Graviera (Greece), and Kefalograviera (Greece). Cheese spoilage, defined as visible colonies of molds or unpleasant organoleptic changes, was determined macroscopically and by sensory tests. A scoring scale with three categories was used. Class 1 corresponds to high-quality cheese without any off-odor or off-flavor, class 2 corresponds to cheese that had slight off-odors or off-flavors but was still acceptable, and class 3 corresponds to cheese of unacceptable quality. The shelf life limit was defined as the point at which 50% of the panelists rejected the cheese samples (*18*).

Experimental Design and Statistical Analysis. In the experiments conducted, the effects of initial cell concentration, cell immobilization, and ripening temperature were studied. All treatments were carried out in triplicate, and the mean data are presented. The standard deviation (SD) was lower than ±0.002 forethanol values and lower than ±0.05 for all other values. The data were subjected to one-way analysis of variance (ANOVA) to test the differences among the results. Coefficients, ANOVA tables, and significance $p < 0.05$ were computed using Origin v.7.0 (OriginLab Corporation, Northampton, MA). Differences between cheese samples in volatile compositions were calculated by dividing the number of differences in volatiles that were determined by the total number of compounds that were detected in both cheese samples.

Figure 2. Cross-section of hard-type cheese after 15 days of ripening, demonstrating the effect on texture and gas hole formation of initial freeze-dried kefir cells (1.0 g/L milk) immobilized on casein.

RESULTS AND DISCUSSION

Freeze-Dried Kefir, Degree of Openness, and Sensory Analysis. Openness in texture, ripening time, and shelf life of hard-type cheeses constitute some of the main parameters affecting cheese quality and may also constitute considerable measures in determination of production costs. Openness in texture can be formed due to mechanical pressure or due to the action of microorganisms that liberate CO_2. Depending on the specifications of the final product, the degree of openness can be characterized as a quality requirement or a defect (*28*). In hard-type cheese production and more precisely in Graviera-type cheeses, openness in texture constitutes a means for consumers to evaluate the ripening process without testing the product. The contribution of kefir, as a starter culture, to the degree of openness in hard-type cheeses was studied by evaluating (i) the initial freeze-dried cell concentration, (ii) the ripening temperature of hard-type cheese, and (iii) the use of immobilized cells on casein. The results are presented in **Figures 1** and **2** and **Table 1**. **Figure 1** shows the effect of initial freeze-dried cell concentration on the degree of openness in hard cheese as compared to rennet cheese. It is clear from our results that

Table 1. Shelf-Life and Sensory Characteristics of Hard-Type Cheeses Made without Starter Culture (Rennet Cheeses) and Cheeses Made with Addition of Free or Immobilized Kefir Cells, During Ripening at Various Temperatures

ripening temp (°C)	cheese sample	shelf life (days)	taste[a]	aroma[a]	textural characteristics[a]	degree of openness[a]
5	rennet cheese	20	2.00	1.98	4.40	1.13
	0.5 g kefir/L milk	>90	6.07	6.10	6.12	6.55
	1.0 g kefir/L milk	>90	6.55	6.67	6.18	6.83
	2.0 g kefir/L milk	>90	5.41	5.64	5.99	6.12
	1.0g immobilized k efir/L milk	70	6.00	6.11	5.90	4.35
18	rennet cheese	7	1.88	2.04	4.37	1.15
	0.5 g kefir/L milk	28	6.00	6.14	6.05	6.51
	1.0 g kefir/L milk	30	6.16	6.73	6.03	6.85
	2.0 g kefir/L milk	15	5.06	5.43	5.96	6.20
	1.0g immobilized k efir/L milk	10	5.90	6.05	5.68	4.30
roomtemp	rennet cheese	90	2.21	2.12	1.00	1.04
	0.5 g kefir/L milk	>90	6.11	6.13	2.12	6.02
	1.0 g kefir/L milk	>90	6.56	6.70	2.21	6.08
	2.0 g kefir/L milk	>90	5.45	5.70	2.19	5.95
	1.0 g immobilized kefir/L milk	90	6.05	6.13	1.10	4.26

[a] Taste, aroma, textural characteristics, and gas hole formation were assessed on a scale of 1−7 (1, unacceptable; 3, objectionable; 5, good; and 7, superior) according to Menéndez et al. (*27*), and mean values are presented.

the three hard-type cheeses produced with freeze-dried kefir as the starter culture led to a substantial increase of openness in the texture. A cell concentration of 1g/L freeze-dried kefir culture appeared to be the most effective in opening the texture as compared to rennet cheese. This sample also was better with regard to texture, color, flavor, and taste as compared to the other three samples (**Figure 1** and **Table 1**). Notably, after 15 days of ripening, an increased degree of openness was observed in all cheeses made using freeze-dried kefir as a starter culture as compared to in rennet cheese. It has been well-demonstrated that immobilized cells of yeasts present an increased metabolic activity as compared to free cells (*29*). Similarly, recent data in our laboratory suggest that immobilized freeze-dried kefir cells on casein are characterized by an increased fermentation activity as compared to free cells (*21*). Furthermore, casein is a component of milk and therefore constitutes a compatible support for cell immobilization in cheese production. Subsequently, the impact of immobilized kefir cells on openness in cheese texture was evaluated after 15 days of ripening. The cheese product, which was made using immobilized kefir cells, was characterized by an increased degree of openness as compared to rennet cheese (**Figure 2**). The effect of ripening temperature and type of starter culture on the degree of openness is shown in **Table 1**. Our results clearly show that the use of a kefir starter culture, immobilized or not, increases significantly ($p < 0.05$) the degree of openness independently of the ripening temperature (**Table 1**). This is a reasonable development as the kefir culture includes, apart from yeasts, lactic acid bacteria that are used in cheese manufacturing for openness development during ripening (*30*). Kefir yeasts also are expected to contribute to gas formation through the alcoholic fermentation of lactose. The increase in openness is also an indication that the ripening in cheeses made with kefir has been processed further as compared to rennet cheese, and therefore, early formation of aromatic compounds may be suggested.

Sensory analysis showed significant differences ($p < 0.05$) among rennet and kefir cheeses in taste, flavor, textural characteristics, and degree of openness. Cheese samples made with 1.0 g kefir/L milk had the highest total score (**Table 1**). Kefir cheeses scored similar values as compared to commercial hard-type cheeses (data not shown), whereas rennet cheese scores were significantly lower ($p < 0.05$). Temperature had a significant effect only on textural characteristics ($p < 0.05$) mainly because of moisture loss at room temperature (**Table 2**).

Table 2. Moisture Content of Hard-Type Cheeses Made without Starter Culture (Rennet Cheeses) and Cheeses Made with Addition of Free or Immobilized Kefir Cells, During Ripening at Various Temperatures

		moisture (%)	
ripening temp (°C)	cheese sample	0 day	90 day
5	rennet cheese	50.32	38.01
	1.0 g kefir/L milk	50.54	37.78
	1.0 g immobilized kefir/L milk	50.45	38.10
18	rennet cheese	50.12	36.80
	1.0 g kefir/L milk	50.11	36.01
	1.0 g immobilized kefir/L milk	50.56	37.18
roomtemp	rennet cheese	50.03	16.05
	1.0 g kefir/L milk	50.06	15.78
	1.0 g immobilized kefir/L milk	50.34	15.27

Effect of Freeze-Dried Kefir Starter Culture on Shelf Life and Physicochemical Characteristics of Hard-Type Cheeses. One of the most important parameters for industrial hard-type cheese production is the shelf life of the final product. The effect of freeze-dried kefir starter culture on the hard-type cheese shelf life was evaluated throughout the ripening period by monitoring the development of visible mold colonies on cheeses produced using various initial cell concentrations of starter culture (**Figures 3** and **4**). Our results indicated that the hard-type cheese produced using 1 g of freeze-dried kefir/L of milk as a starter culture exhibited a higher resistance to mold formation than cheeses made without starter culture or with a lower or higher cell concentration of starter culture (**Figure 3** and **Table 1**). However, a relatively high resistance, but limited as compared to the aforementioned optimum, also was observed when the same concentration of freeze-dried kefir cells (1 g/L milk) immobilized on casein was used as a starter culture in cheeses ripened at 5 °C (**Figure 4** and **Table 1**). However, cheeses made with immobilized kefir culture and ripened at 18 °C exhibited a relatively short shelf life (**Table 1**). The increase in resistance to mold development and the extension of the shelf life of the final products ripened at 5 °C (**Table 1**) may be attributed to the relatively rapid pH drop (**Table 3**) caused by lactic acid fermentation that is accelerated by the starter culture. It also may be a consequence of bacteriocin formation by one or more strains present in the multistrain culture of kefir (*31*). The increased shelf life that was observed in cheeses ripened at room temperature is possibly attributed to the very low moisture content of the final products (**Table 2**). The acidity (as g of lactic acid/100 g of cheese) of rennet cheese was significantly lower ($p < 0.05$) than the other types of cheeses ripened at the same temperature (**Table 3**). The ripening temperature affected significantly ($p < 0.05$) the acidity of all the tested cheese samples with the exception of rennet cheese as results were similar for all ripening temperatures used. The pH values of rennet and kefir cheeses were significantly affected ($p < 0.05$) by the ripening temperature and by the use of free or immobilized cells. Free cells of kefir as a starter culture showed a higher rate of pH decrease and a lower final pH value (4.96) as compared to the other types of cheeses. Decreases in sugar and pH and an increase in lactic acid occurred at lower rates at low ripening temperatures (**Table 3**).

Effect of Freeze-Dried Kefir Starter Culture on Microbial Associations of Hard-Type Cheeses. The evolution of yeasts,

Figure 3. Digital scans of hard-type cheeses, after 30 days of ripening, demonstrating the effect of the initial concentration of freeze-dried kefir starter culture on the formation of mold: (**A**) rennet cheese, (**B**) 0.5 g kefir/L milk, (**C**) 1.0 g kefir/L milk, and (**D**) 2.0 g kefir/L milk.

Figure 4. Digital scan of hard-type cheeses, after 30 days of ripening, demonstrating the effect of the initial concentration of 1.0 g of freeze-dried immobilized kefir starter culture per liter of milk on the formation of mold.

lactobacilli, lactococci, and total aerobic counts was assayed in hard-type cheeses ripened at various temperatures. Our results (**Table 4**) indicated that the initial concentration of all microbial taxa tested was greater than 7.17 log CFU/g in cheeses made using the kefir culture, while it was less than 3.84 log CFU/g in rennet cheese. Apart from lactococci, all microbial counts in rennet cheese were significantly ($p < 0.05$) affected by the ripening temperature. On the other hand, no differences were observed in microbial counts of cheeses with 1.0 g kefir culture/L milk, ripened at different temperatures. The ripening temperature affected significantly ($p < 0.05$) the total aerobic count as well as yeast and mold in cheeses with 1.0 g of immobilized kefir culture/L of milk. Generally, the addition of the kefir culture in cheese samples significantly increased all microbial counts. After the first increase in microbial counts that was observed on the 15th day of ripening, all counts apart from lactobacilli began to decrease by higher rates in kefir cheeses. Indeed, lactobacilli counts increased in all cheeses, reaching levels greater than 8.50 log CFU/g when free cells of kefir were used and greater than 9.10 log CFU/g when immobilized kefir culture on casein was used (**Table 4**). When these taxonomic groups were assayed in Feta-type unsalted cheese made using the same culture, a significant increase was observed during ripening or storage (*18*). This is possibly due to the lack of salt and its effect on the moisture content. All cheeses made in this study were characterized by a moisture content of <39% after 90 days of ripening, while cheeses ripened at room temperature had moisture contents even less than 17%. This low moisture content is possibly the reason for the only observed decrease in lactobacilli counts made in cheeses ripened at room temperature between the 60th and the 90th day of ripening. The type of starter culture did not seem to affect the rate of moisture loss significantly as the moisture levels at the end of the ripening period were similar for all types of cheeses that were ripened at the same temperature (**Table 2**).

Effect of Freeze-Dried Kefir Starter Culture on Flavor of Hard-Type Cheeses. Flavor constitutes one of most important characteristics in hard-type cheeses, affecting their value and market destination (*32*). Volatile compounds that are formed via proteolysis, lipolysis, and carbohydrate degradation during ripening constitute the main key substances that determine cheese flavor (*33*). GC/MS analysis was employed to determine volatile compounds in cheeses made with the freeze-dried kefir culture (1 g kefir/L milk) and ripened for 90 days either at 5 or 18 °C, given that these cheeses were considered to be the most attractive with regard to shelf life, textural characteristics, taste, and aroma (**Table 1**). Control cheeses made only with rennet also were analyzed (**Table 5**). A total of 47 compounds of esters, organic acids, alcohols, and carbonyl compounds were detected: 25 in rennet cheese ripened at 18 °C, 38 in kefir cheese ripened at 18 °C, 20 in rennet cheese ripened at 5 °C, and 31 in kefir cheese ripened at 5 °C. The main contribution of kefir, with regard to changes in concentration, was observed in fatty acids (422% increase), which are important components of cheese flavor and may originate either from milk fat lipolysis or from the breakdown of amino acids (*34*). Other important contributions of kefir were observed in the composition of alcohols and carbonyl compounds, especially in cheeses ripened at 5 °C. Indeed, differences in carbonyl and alcohol composition between kefir and rennet cheeses ripened at 5 °C were at levels of 75%, while the corresponding differences in cheeses ripened at 18 °C were 42 and 64%. The main carbonyl compounds associated with the use of kefir in this study were benzaldehyde and butanal, although the latter only was detected in kefir cheese ripened at 5 °C. Both aldehydes have been shown to occur in ripened hard-type cheeses such as Gouda, Gruyère, Edam, and Parmesan (*33*). Straight-chain aldehydes, such as butanal, are formed in ripened cheeses through β-oxidation of unsaturated fatty acids (*35*). Among the alcohols associated with the use of kefir in this study, only 2-hexanol appears to be a common component in other cheeses such as Cheddar, Emmental, Comté, Camembert, Provolone, and Parmesan (*33, 36*). None of the other alcohols was detected in the studies or in two other cheeses (Feta-type and whey cheese) produced using the same culture as a starter (*18, 19*). Alcohols are considered to be derived mainly from

Table 3. Residual Sugar, Ethanol, and Acidity of Hard-Type Rennet Cheeses and Cheeses Made with 1 g/L Freeze-Dried Kefir Starter or 1 g/L Freeze-Dried Kefir Starter Immobilized on Casein, During Ripening at Various Temperatures

cheese sample	ripening temp (°C)	ripening time (days)	lactose (g per 100 g cheese)[a]	glucose (g per 100 g cheese)[a]	galactose (g per 100 g cheese)[a]	ethanol (g per 100 g cheese)[a]	pH	acidity as lactic acid (g per 100 g cheese)
rennet cheese		0	1.84	Nd	Nd	0.02	6.58	0.11
	5	15	1.00	Nd	Nd	Nd	5.75	0.41
		30	0.81	Nd	Nd	0.01	5.24	0.56
		60	0.82	Nd	Nd	0.01	5.49	0.20
		90	0.81	Nd	Nd	Nd	5.48	0.23
	18	15	0.98	Nd	Nd	0.03	5.70	0.41
		30	0.45	Nd	Nd	0.01	5.26	0.52
		60	0.41	Nd	Nd	Nd	5.45	0.29
		90	0.39	Nd	Nd	Nd	5.46	0.25
	room temp	15	1.09	Nd	Nd	0.00	5.72	0.41
		30	1.04	Nd	Nd	0.02	5.22	0.59
		60	0.82	Nd	Nd	0.01	5.49	0.20
		90	0.69	Nd	Nd	Nd	5.47	0.25
1.0 g kefir/L milk		0	0.65	0.77	0.60	0.01	6.53	0.16
	5	15	Nd	Nd	Nd	0.04	5.05	0.73
		30	Nd	Nd	Nd	0.02	5.04	0.83
		60	Nd	Nd	Nd	Nd	4.99	0.90
		90	Nd	Nd	Nd	Nd	5.02	0.83
	18	15	Nd	Nd	Nd	0.01	5.01	0.76
		30	Nd	Nd	Nd	0.02	5.11	0.68
		60	Nd	Nd	Nd	0.01	5.27	0.50
		90	Nd	Nd	Nd	Nd	5.13	0.63
	Room temperature	15	Nd	Nd	Nd	0.01	4.99	0.90
		30	Nd	Nd	Nd	0.01	4.96	1.00
		60	Nd	Nd	Nd	Nd	4.94	1.17
		90	Nd	Nd	Nd	Nd	4.96	1.01
1.0 g immobilized kefir/L milk		0	1.48	0.51	0.47	0.01	5.87	0.34
	5	15	Nd	Nd	Nd	0.01	5.22	0.58
		30	Nd	Nd	Nd	Nd	5.31	0.51
		60	Nd	Nd	Nd	Nd	5.29	0.52
		90	Nd	Nd	Nd	Nd	5.22	0.57
	18	15	Nd	Nd	Nd	0.01	5.11	0.70
		30	Nd	Nd	Nd	Nd	5.25	0.55
		60	Nd	Nd	Nd	0.01	5.26	0.54
		90	Nd	Nd	Nd	Nd	5.19	0.61
	room temp	15	Nd	Nd	Nd	Nd	5.07	0.73
		30	Nd	Nd	Nd	0.01	5.24	0.55
		60	Nd	Nd	Nd	Nd	5.19	0.60
		90	Nd	Nd	Nd	Nd	5.20	0.58

[a] Nd: not detected.

Table 4. Microbial Counts in Hard-Type Rennet Cheeses and Cheeses Made with 1 g/L Freeze-Dried Kefir Starter or 1 g/L Freeze-Dried Kefir Starter Immobilized on Casein, During Ripening at Various Temperatures

		total aerobic counts (log CFU/g)			yeasts and molds (log CFU/g)			lactococci (log CFU/g)			lactobacilli (log CFU/g)		
ripening temp (°C)	ripening time (days)	rennet	1.0 g kefir/L milk	1.0 g immobilized kefir/L milk	rennet	1.0 g kefir/L milk	1.0 g immobilized kefir/L milk	rennet	1.0 g kefir/L milk	1.0 g immobilized kefir/L milk	rennet	1.0 g kefir/L milk	1.0 g immobilized kefir/L milk
	0	3.78	7.17	7.70	3.84	7.78	7.48	3.48	7.34	7.85	3.00	7.26	7.73
5	15	6.53	9.12	9.52	6.38	9.09	9.21	3.69	7.30	7.47	6.18	7.94	8.04
	30	6.17	8.90	9.45	6.17	8.32	9.58	3.00	6.88	7.03	6.79	7.99	8.17
	60	6.00	8.72	9.30	5.47	8.31	9.54	2.89	5.99	6.75	6.94	8.48	9.06
	90	5.99	8.17	9.14	5.37	8.05	9.19	2.77	5.10	6.02	6.90	8.63	9.17
18	15	6.84	8.82	9.45	5.79	8.66	9.13	3.78	7.28	7.41	5.83	7.71	8.12
	30	6.83	8.21	9.30	5.69	8.15	9.29	3.07	6.76	6.90	6.57	7.95	8.55
	60	6.37	8.06	9.21	5.47	8.11	9.21	3.00	5.89	6.87	6.80	8.46	9.16
	90	6.12	8.08	8.99	5.28	7.99	8.96	2.90	5.11	6.35	6.87	8.60	9.29
room temp	15	6.61	8.81	9.33	5.95	9.00	9.22	3.69	7.15	7.43	6.05	7.87	8.00
	30	6.52	8.66	9.10	5.80	8.30	8.88	3.11	6.72	7.00	6.61	7.92	8.53
	60	6.29	8.50	9.02	5.07	8.15	8.51	3.02	5.71	6.41	6.89	8.50	8.60
	90	5.89	7.65	7.69	5.00	7.69	7.30	2.60	4.73	6.00	5.63	7.66	7.45

the corresponding aldehydes that themselves are produced from amino acids and/or fatty acids or also may be derived from ketones that mainly come from fatty acids (*33*). Thus, this radical change that was detected in alcohols could be associated with the significant increase in fatty acids that was observed in cheeses made with kefir. A significant difference (64%) also was observed in ester composition in cheeses ripened at 5 °C. Phenylethyl acetate, detected in kefir cheese samples and in rennet cheese ripened at 5 °C, is known for its floral, rose-like aroma and was determined to be the major odorant in Camembert (*37*). Other esters that were associated with kefir culture use in this study are characterized by a fruity aroma (*38, 39*). The increase in composition and quantity of esters, carbonyl compounds, fatty acids, and alcohols that were observed in kefir cheeses is a consequence of a complex series of microbiological, biochemical, and chemical processes occurring during ripening.

Technological Considerations. Lactobacilli have gained a substantial role in the dairy industry due to their involvement in the improvement of cheese flavor and the production of substances with health promoting properties. Given the general concern with regard to the viability of such cultures at the time of consumption to be effective, products with a short shelf life

Table 5. SPME-GC/MS Analysis of Aroma-Related Compounds (μg/kg Cheese) Isolated from Rennet Cheese and Cheese Made Using 1 g of Freeze-Dried Kefir/L of Milk as a Starter Culture after a 90 Day Ripening Period at 18 and 5 °C

		ripened at 18 °C		ripened at 5 °C	
compound	identification method[a]	rennet cheese	kefir cheese	rennet cheese	kefir cheese
		Esters			
ethyl butyrate	RT, KI, MS	0.17	1.69	0.13	Nd
ethyl hexanoate	RT, KI, MS	1.35	3.56	1.10	4.32
ethyl octanoate	RT, KI, MS	4.51	10.27	3.47	4.92
ethyl nonanoate	RT, MS	0.08	Nd	Nd	4.73
ethyl decanoate	RT, MS	5.71	7.24	5.26	10.53
ethyl-9-decenoate	MS	Nd	Nd	Nd	2.41
ethyl-9-hexadecenoate	MS	0.74	0.86	Nd	0.93
ethyl dodecanoate	KI, MS	Nd	0.04	Nd	2.71
ethyl tetradecanoate	KI, MS	Nd	0.02	Nd	0.08
ethyl pentadecanoate	MS	1.73	2.04	1.35	Nd
2-ethylhexyl-isobutanoate	MS	Nd	2.71	Nd	Nd
phenyl ethyl acetate	MS	Nd	1.79	1.21	2.38
total esters		14.29	30.22	12.52	33.01
		Organic acids			
acetic acid	KI, MS	3.61	4.67	Nd	3.92
butanoic acid	KI, MS	Nd	9.31	Nd	9.12
pentanoic acid	MS	Nd	1.34	Nd	Nd
hexanoic acid	KI, MS	0.05	4.21	0.02	6.58
heptanoic acid	KI, MS	3.64	3.32	Nd	4.02
octanoic acid	RT, KI, MS	8.64	12.73	5.65	6.26
octadecanoic acid	KI, MS	0.01	0.06	0.07	0.04
nonanoic acid	RT, KI, MS	0.34	1.36	Nd	6.35
decanoic acid	RT, KI, MS	3.22	3.51	2.37	2.90
dodecanoic acid	KI, MS	1.10	2.31	1.06	2.57
tetradecanoic acid	KI, MS	2.7	3.4	10.1	39.7
total organic acids		23.31	46.22	19.27	81.46
		Alcohols			
ethanol	RT, KI, MS	10.34	12.61	10.08	12.44
2-hexanol	MS	Nd	0.02	Nd	0.02
2-ethyl-1-hexanol	RT, MS	1.06	6.83	Nd	Nd
3-methyl-1-butanol	RT, MS	0.13	Nd	0.12	Nd
3-methyl-2-buten-1-ol	MS	Nd	Nd	Nd	1.05
2-undecanol	MS	Nd	1.83	0.25	2.06
2-nonanol	MS	0.03	2.64	Nd	0.12
2,3-butandiol	KI, MS	0.75	0.81	1.60	Nd
1,3-butandiol	KI, MS	1.38	Nd	1.05	Nd
2-ethyl-1-dodecanol	MS	Nd	Nd	Nd	0.08
nonanol	RT, MS	Nd	0.01	Nd	Nd
1-tridecanol	KI, MS	Nd	0.07	Nd	0.10
1-hexadecanol	MS	Nd	0.01	Nd	Nd
2-hexyl-1-octanol	MS	Nd	0.24	Nd	Nd
2-propyl-1-heptanol	MS	Nd	Nd	Nd	0.07
phenyl ethanol	RT, KI, MS	3.31	4.59	3.75	4.31
phytol	MS	Nd	0.05	Nd	Nd
total alcohols		17.00	29.71	16.85	20.25
		Carbonyl compounds			
3-hydroxy-2-butanone	MS	Nd	2.94	0.67	2.57
2-heptanone	RT, MS	0.06	1.05	Nd	Nd
2-undecanone	KI, MS	0.06	0.73	Nd	Nd
8-nonen-2-one	MS	Nd	0.89	Nd	Nd
nonanal	KI, MS	Nd	Nd	0.06	Nd
benzaldehyde	KI, MS	Nd	1.14	Nd	1.05
2-butenal	KI, MS	Nd	Nd	Nd	0.02
total carbonyl compounds		0.12	3.81	0.06	1.07

[a] RT: Positive identification by retention times that agree with authentic compounds and by mass spectra of authentic compounds generated in the laboratory; KI: tentative identification by Kovats' retention index; MS: positive identification by mass spectra obtained from NIST107, NIST21, and SZTERP libraries; and Nd: not detected.

have gained most of the attention. Our results show that the kefir culture, especially when immobilized on casein, either promotes the propagation of lactobacilli in hard-type cheeses during ripening or contains lactobacilli strains capable of growing in this environment. The relative increase of lactobacilli, which was associated with the addition of kefir (**Table 4**), was correlated to the total reduction of lactose and the increase of lactic acid (**Table 3**). The consequent pH drop observed in the final products may be related to the increased resistance against spoilage and the increased shelf life. Furthermore, freeze-dried kefir, immobilized or not, improved the flavor and texture characteristics and increased the degree of openness in comparison to traditional hard-type cheese products. Kefir cells immobilized on casein failed to extend the shelf life of the final product at the same level as free cells, although similar counts of microbial associations were determined in both cheeses (**Table 4**). The kefir culture had, however, a major effect on the profile of volatiles of cheeses ripened for 90 days at 5 °C, changing significantly the composition of alcohols, carbonyl compounds, and esters and increasing the concentration of organic acids. Given the low production cost of kefir cultures that was demonstrated recently in a pilot plant operating under industrial conditions (*40*) and the advantages of kefir regarding flavor, taste, shelf life, and possibly as a probiotic agent of cheese (*18, 19*), freeze-dried kefir cells may constitute a valuable tool in the cheese industry as a starter culture.

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