

Microbiological and chemical properties of kefir manufactured by entrapped microorganisms isolated from kefir grains

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ABSTRACT

In this study, various yeasts (*Kluyveromyces marxianus*, *Saccharomyces turicensis*, *Pichia fermentans*) and lactic acid bacteria (*Lactobacillus kefiranofaciens*, *Lactobacillus kefir*, *Leuconostoc mesenteroides*) were entrapped in 2 different microspheres using an entrapment ratio for the strains that was based on the distribution ratio of these organisms in kefir grains. The purpose of this study was to develop a new technique to produce kefir using immobilized starter cultures isolated from kefir grains. An increase in cell counts with fermentation cycles was observed for both the lactic acid bacteria (LAB) and yeasts, whereas the cell counts of kefir grains were very stable during cultivation. Scanning electron microscopy showed that the short-chain lactobacilli and lactococci occupied the surface of the LAB microspheres, whereas the long-chain lactobacilli were inside the microspheres. When the yeasts were analyzed, cells at a high density were entrapped in cracks on the surface and within the microspheres, where they were surrounded by the short-chain lactobacilli. The distribution of the LAB and yeast species in kefir produced from grains and microspheres showed that there was no significant difference between the kefirs produced by the 2 methods; moreover, *Leu. mesenteroides* and *K. marxianus* were the predominating microflora in both types of kefir. There was no significant difference in the ethanol and exopolysaccharide contents between the 2 kefirs, although the acidity was different.

Key words: entrapment, kefir grains, kefir

INTRODUCTION

Kefir is an acidic and mildly alcoholic fermented dairy product that is believed to contain many functional

substances. Kefir differs from other fermented dairy products in that the milk is fermented by the presence of a mixed group of microflora confined to a matrix of discrete “kefir grains,” which are recovered subsequent to fermentation (Marshall and Cole, 1985). Kefir culture seems to have potential in the fabrication of a range of value-added products including whey cheese (Dimitrellou et al., 2007), Feta-type cheese (Kourkoutas et al., 2006), whey-based alcoholic beverages (Athanasiadis et al., 2004; Assadi et al., 2008; Dimitrellou et al., 2008), potable alcohol (Athanasiadis et al., 2002; Kourkoutas et al., 2002), and baker’s yeast (Plessas et al., 2005).

Modern kefir grain production is based on continuous cultivation in milk, resulting in biomass increase of 5 to 7% per day (Libudzisz and Piatkiewicz, 1990), but kefir grains can only grow from pre-existing grains (Schoevers and Britz, 2003). Motaghi et al. (1997) produced kefir grains in a goat-hide bag using pasteurized milk inoculated with sheep intestinal flora, followed by culture on the surface of milk. Despite intensive research and many attempts to produce kefir grains from the pure or mixed cultures that are normally present in the grains, no successful results have been reported to date (Liu and Moon, 1983; Libudzisz and Piatkiewicz, 1990). This failure can probably be ascribed to the fact that very little is known about the mechanism of grain formation. It is likely that a combination of different factors influences the biomass increase of the kefir grains, including renewal of the milk at regular intervals, cultivation temperature, grain washing, and the presence of essential nutrients at the correct concentrations in the growth medium. Schoevers and Britz (2003) found the highest grain increase occurred when activated grains were cultivated with agitation at 25°C in low-fat milk containing tryptose and with the fermented milk replaced daily. However, the biomass increase of the grains was still too small and too slow to produce kefir commercially. In addition, a stable and constant starter culture would be necessary for manufacturing a quality kefir beverage and this is difficult to sustain because of the complex microbiological composition of the grains.

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Several processes have been developed to produce a kefir-like beverage in which no grains are used. In Russia, a mother culture is prepared by carrying out traditional kefir fermentation and sieving the grains. About 1 to 3% of this mother culture is added to pasteurized milk (Farnworth and Mainville, 2003). The use of defined cultures to produce kefir is also in progress as a way of standardizing kefir production (Duitschaever et al., 1988; Beshkova et al., 2002), and this approach may eliminate the problems associated with the use of kefir grains (Duitschaever et al., 1987). The use of the specific microflora isolated from kefir grains as a starter culture can produce a product whose properties are close to those of fine traditional kefir. However, the quality of these kefir products is significantly different from that of kefir fermented with grains (Farnworth and Mainville, 2003). Organoleptic tests have shown that the kefir produced with kefir grains is more desirable compared with kefir produced with a starter culture (Assadi et al., 2000). Beshkova et al. (2002) explained that the microflora of a kefir grain is in symbiotic equilibrium and that the species and quantitative structure of the various groups of microorganisms change significantly along the pathway from kefir grains to kefir and then on to a second batch of kefir.

Kefir production by kefir grains is difficult to put into practice, but the kefir produced by this method is of better quality compared with kefir produced by grain-free methods. A new method needs to be developed to maximize kefir production. Immobilization of kefir microorganisms may provide a solution to the large-scale production of kefir with a similar quality to that of fermented grain kefir. Among the available techniques for immobilizing living cells, entrapment in calcium alginate beads has been frequently used for the immobilization of microorganisms (Sheu and Marshall, 1993). This technique has been anticipated in production of ethanol from beet molasses (Göksungur and Zorlu, 2001), acceleration of cheese ripening (Desmond et al., 2002), improvement of probiotic survival (Chen et al., 2007), and continuous ethanol fermentation (Nagashima et al., 1984).

Thus, the purpose of this study was to develop a new technique to produce kefir using entrapped starter cultures based on the yeast and bacterial strains isolated from kefir grains. In this research, kefir starter cultures previously isolated from kefir grains in our laboratory were immobilized in alginate microspheres. The encapsulation ratio of the strains was based on the strains' distribution ratio in kefir grains (Chen et al., 2008; Wang et al., 2008). The microbiological properties of the microspheres were investigated. In addition, because kefir quality depends on properties such as chemical composition, microflora, rheology, and

organoleptic features, we also evaluated the microbial and chemical properties of the kefir produced by the entrapped cultures and then compared these with kefir made from kefir grains.

MATERIALS AND METHODS

Microorganisms and Growth Conditions

Kefir grains collected from Taiwan were evaluated in this study. In the laboratory, kefir grains were inoculated (5%, wt/vol) and propagated in sterilized milk at 20°C for 20 h. The grains were retrieved by sieving, reinoculated into fresh pasteurized milk (National Taiwan University Experimental Dairy Farm, Taipei, Taiwan), and incubated again at 20°C for 20 h.

Various lactic acid bacteria (*Lactobacillus kefirano-faciens*, *Lactobacillus kefir*, and *Leuconostoc mesenteroides*) and yeasts (*Kluyveromyces marxianus*, *Saccharomyces turicensis*, and *Pichia fermentans*) had been previously isolated from kefir grains and identified by PCR-denaturing gradient gel electrophoresis (DGGE) and DNA sequencing (Chen et al., 2008; Wang et al., 2008) in our laboratory. All lactic acid bacteria (LAB) were cultured separately using Lactobacilli de Man, Rogosa, and Sharpe (MRS) broth (Difco Laboratories, Detroit, MI) incubated under aerobic conditions at 30°C. The yeasts were cultured separately in potato dextrose broth (Difco) at 25°C. Each culture was harvested after 24 h by centrifugation (3,000 × *g*, 10 min at 4°C), washed and resuspended twice in saline solution. The final cell counts for each kefir grain microorganism were then adjusted to 10⁹ cells/mL.

Entrapment of Kefir Culture Starters

In this study, the yeasts (*Klu. marxianus*, *S. turicensis*, *P. fermentans*) and LAB strains (*L. kefirano-faciens*, *L. kefir*, *Leu. mesenteroides*) were entrapped in 2 different microspheres, in which the entrapment ratio of the strains was based on the distribution ratio found in kefir grains (Chen et al., 2008; Wang et al., 2008). Microspheres were prepared by mixing 1% (vol/vol) of either the LAB strain mixture or the yeast mixture with 2% sodium alginate (Sigma, St. Louis, MO). An extrusion entrapment technique was then used (Chen et al., 2007). After washing, 1% (vol/vol) of the culture concentrate was mixed with 50 mL of gel-coating solution that had been sterilized at 121°C for 15 min. The cell suspension was injected through a 0.11-gauge needle into sterile 0.1 M CaCl₂. The beads were approximately 0.5 mm in diameter and were allowed to stand for 1 h to allow solidification. They were then rinsed with and subsequently stored in sterile 0.1% peptone solution at 4°C.

Production of Kefir

The kefir using the entrapped culture (microsphere kefir) was produced by adding 10% of the entrapped kefir culture starter (5% of the yeast microspheres and 5% of the LAB microspheres) to pasteurized milk at 20°C. The microspheres were sieved and transferred to fresh pasteurized milk every 24 h for 4 wk. Similarly, traditional kefir was produced by inoculating 10% (wt/vol) kefir grains into fresh pasteurized milk at 20°C. The fresh pasteurized milk (3.5% fat, 3.3% protein, and 4.5% lactose) was from National Taiwan University Experimental Dairy Farm (Taipei, Taiwan). After 24 h of incubation, the kefir grains were removed and transferred to fresh pasteurized milk. This procedure was repeated 28 times with both the entrapped and traditional kefir cultures.

Microbiological Analysis

Determination of Yeast and LAB Cell Counts.

To study the effect of the consecutive fermentation cycles on the cell counts of the LAB and yeast within the entrapped kefir starter culture and kefir grains as well as the kefir produced by entrapped kefir starter culture or kefir grains, the numbers of viable LAB and yeasts expressed as colony-forming units per milliliter (cfu/mL) were estimated. Serial dilutions of each sample were plated in triplicate and the plates were incubated at 30°C (for LAB) and 25°C (for yeast) until colonies formed. The results were the means of 6 counts at 3 different dilutions. The entrapped cells were released from the microspheres according to the method of Sheu and Marshall (1993). For kefir grains, 10 g of grains was suspended in 90 g of sterile saline buffer (0.85% NaCl) and homogenized with a stomacher (Laboratory Blender Stomacher 400, Seward, London, UK) for 20 min.

Calculation of Encapsulation Efficiency

The encapsulation efficiency was calculated based on the modified version of the method of Lin et al. (2005) as follows:

$$\text{Encapsulation efficiency (\%)} = \frac{\text{Encapsulated cells}}{\text{Total cells}} \times 100, \quad [1]$$

where total cells is the cell count of microorganisms in the alginate blended with kefir culture starter and encapsulated cells is the cell count of the entrapped cells released from the microspheres to the buffer. The

encapsulation efficiencies of LAB and yeast were 83 and 82%, respectively.

Determination of Strain Distribution. To determine the distribution of the LAB and yeasts, both the microcapsule kefir and traditional kefir strains underwent proliferation and isolation in MRS agar. The Harrison disc method, adopted from Harrigan (1998), was performed to calculate the distribution of various microorganisms present in a sample. At least 10% of the total colonies were selected from each plate with 30 to 300 colonies and transferred to MRS broth (Difco) for further identification by PCR-DGGE and 16S rDNA sequencing. The PCR-DGGE and sequencing procedures followed the methods described by Chen et al. (2008) and Wang et al. (2008).

Chemical Analysis

Lactic acid was analyzed by HPLC according to the procedure described by Chen et al. (2006). Alcohol was determined by enzymatic methods as described by Beshkova et al. (2002) using K-EtOH kits (Megazyme International Ireland Limited, Wicklow, Ireland). The exopolysaccharide (EPS) assay was carried out by using the modified ethanol precipitation method according to the procedure described by Goh et al. (2005), which was originally developed by Cerning et al. (1994). Productivity of lactic acid and ethanol was defined as the grams of lactic acid or ethanol/volume produced per hour (Papapastolou et al., 2008).

Scanning Electron Microscopy

The microstructures of the microspheres and kefir grains were observed by scanning electron microscopy according to the method of Lin et al. (1999). The beads were fractured and opened using a surgical knife under microscopy. Samples were fixed in 30 g/L glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) at 25°C for 4 h. The samples were then washed in 3 changes of buffer and post-fixed with 10 g/L osmium tetroxide in the same buffer at 25°C for 1 h. After washing in distilled water, the samples were dehydrated in an ethanol series: 15, 30, 50, and 70% for 10 min each; 85 and 95% for 15 min each; and 100% for 1 h. The resulting specimens were then critical-point dried (CO₂ Critical Point Dryer Samdri-PVT-3B, Tousimis, Rockville, MD). Eventually, the samples were fixed in stubs on double-faced metallic tape, covered with a fine layer of gold (Ion Coater JJFC1100E; Jeol Ltd., Tokyo, Japan) while applying a current of 40 mA, and observed by scanning electron microscopy (JSM-6300, Jeol Ltd.).

Statistical Analysis

Statistical analysis was performed using the ANOVA GLM procedure, and Duncan's multiple range test was implemented using the SAS software package (SAS Institute Inc., Cary, NC). Statistical significance was judged at the level $P < 0.05$. Experiments were performed 3 times.

RESULTS AND DISCUSSION

Effect of Entrapment on Kefir Cultures

To understand the microbiological changes in microspheres during fermentation cycles, the viable counts of lactic acid bacteria and yeasts in microspheres were analyzed. An increase in cell counts with the number of fermentation cycles was observed in the microspheres for both LAB (10^7 to 10^{12} cfu/g) and yeasts (10^5 to 10^7 cfu/g), whereas the cell counts in the kefir grains were very stable during cultivation (LAB: 10^9 cfu/g; yeast: 10^7 cfu/g).

The number of entrapped viable cells in gel beads depends on cellular reaction kinetics, internal and external mass transfer of substrates and inhibition products, and on cell growth and release from the bead to the bulk medium (Lacroix et al., 2005). Alginate gel without living cells shows a diffusion coefficient for low-molecular-weight substances similar to that in water (Gosmann and Rehm, 1988; Chai et al., 2004), allowing the free diffusion of low-molecular-weight compounds such as nutrients and metabolic products. The diffusion rate decreases with increasing concentration of microorganisms within the gel matrix according to the exclusion effect. In addition, it was reported in the literature (Arnaud et al., 1992; Masson et al., 1994; Cachon et al., 1998) that entrapped cells could grow and colonize in the beads. Different parameters may influence the cell growth and releases from the bead surface to the bulk medium during fermentation including the type of microorganism and growth characteristics and the composition of gel beads. Other fermentation parameters such as temperature, pH, medium composition, and dilution rates greatly influence cell growth and consequently cell releases from the peripheral layer of beads to the fermentation medium (Lacroix et al., 2005). The characteristics of kefir grains are different from those of gel beads. The grain biomass, which contains several different bacteria and yeasts embedded in a complex matrix of protein and carbohydrate, increases by about 5 to 7% per day during continuous cultivation in milk (Libudzisz and Piatkiewicz, 1990; Schoevers and Britz, 2003).

Lower yeast concentrations in both kefir grains and microspheres compared with immobilized LAB was also observed in this study. The oxygen consumption of yeasts was higher compared with LAB. Gosmann and Rehm (1988) indicated that yeast cells in alginate could not be sufficiently supplied with oxygen at high cell concentrations. The increasing CO_2 concentration due to alcoholic fermentation may have accelerated the decrease of the oxygen concentration in alginate. Lack of oxygen in the beads could be the reason limiting the propagation of yeast.

The effect of fermentation cycles on the microbiological status of the microspheres was also evaluated by scanning electron microscopy. The microspheres without cultivation were spherical [Figure 1a (panel 1) and Figure 2a (panel 1)] with groups of entrapped cells, which was surrounded by matrix, evident in the internal voids [Figure 1a (panel 3) and Figure 2a (panel 3)]. Few LAB or yeast cells were entrapped on the surface of capsules [Figure 1a (panel 2) and Figure 2a (panel 2)]. Skjak-Brak et al. (1989) reported that alginate microparticles usually have a core because of the capsule's heterogeneous gelation mechanism. Both the LAB and yeast microparticles retained their shape, but seemed to be larger, and have a rougher surface and higher porosity after 28 batch fermentation cycles (Figure 1 and Figure 2). Gosmann and Rehm (1988) and Audet et al. (1991) indicated that microorganisms are able to produce organic acids that change the alginate network.

When the LAB microspheres were examined (Figure 1), aggregation of cells within the voids resulted in complete filling of the outer and inner pores of the microparticles. The very high viable immobilized biomass found in our study is in agreement with our previous viable counts of LAB and data reported in the literature for immobilized LAB by Bergmaier et al. (2005) using ImmobaSil (Cellon SA, Bereldange, Luxembourg). After 14 fermentation cycles [Figure 1b (panel 2)], the short-chain lactobacilli and lactococci were found to occupy the surface and the long-chain lactobacilli were found inside the microspheres [Figure 1b (panel 3)]. However, a morphological change in the LAB was observable after 28 fermentation cycles. The surface of the particle had become porous and was packed with a high density of short-chain lactobacilli; furthermore, short rods instead of long rods filled the center of the capsules. Cell adaptation is often accompanied by significant changes in cell morphology as a response to environmental perturbations (Ye et al., 1999; Jan et al., 2001). Morphological changes have also been observed with immobilized *Lactobacillus rhamnosus* (Bergmaier et al., 2005) and *Propionibacterium acidipropionici*

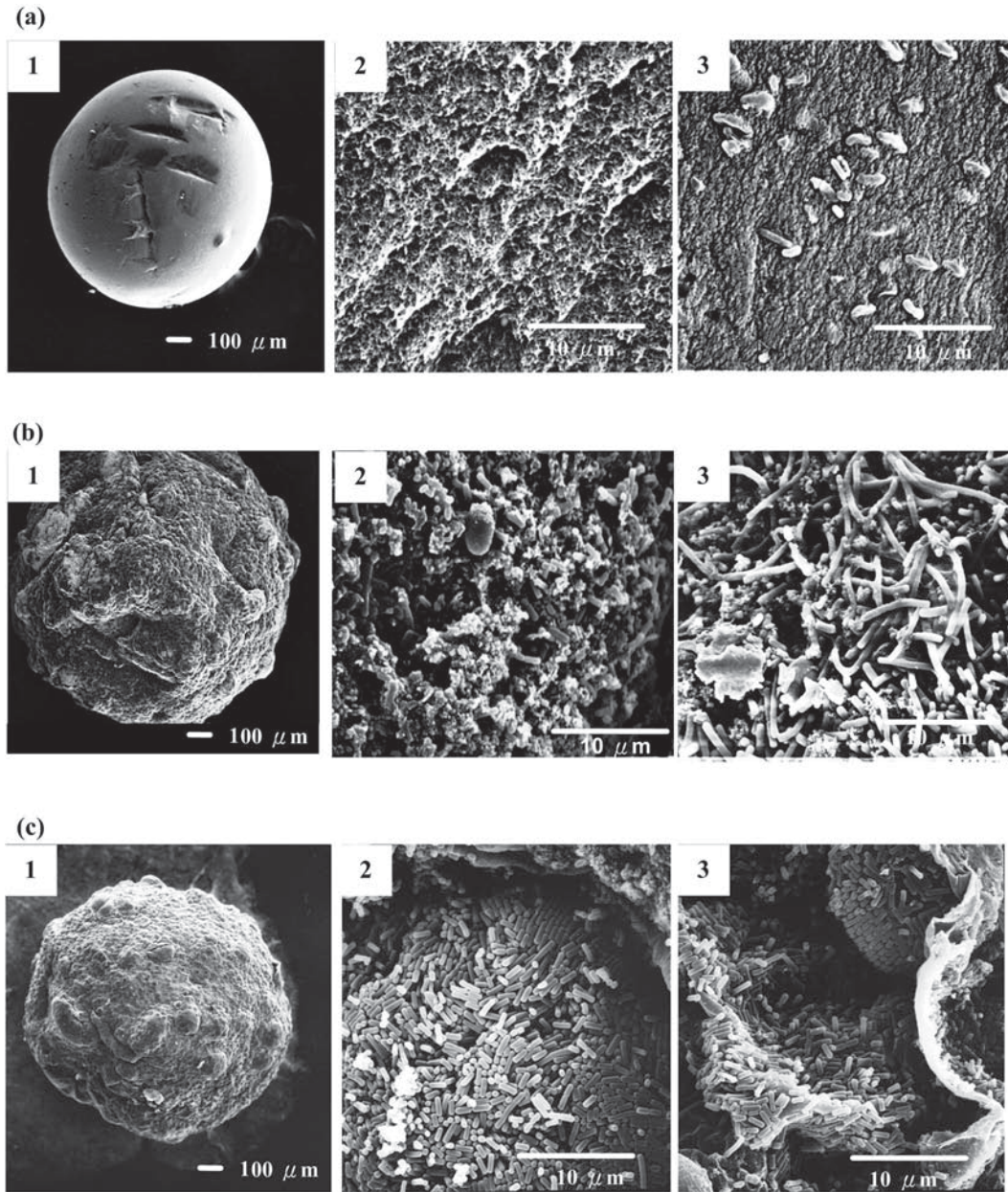


Figure 1. Scanning electron micrograph showing the effect of number of fermentation cycles on the entrapped lactic acid bacteria: (a) 0 cycles; (b) 14 cycles; (c) 28 cycles. Panel 1 = whole gel bead; 2 = bead surface; 3 = bead cross-section.

(Suwannakham and Yang, 2005) after continuous fermentation. In the high-cell-density environment with relatively low nutrient and high acid concentrations, the observed morphological change would be a natural response for cells to adapt and survive (Suwannakham and Yang, 2005).

When the yeast microspheres were examined after 28 fermentation cycles, a high density of yeast cells was found entrapped in cracks on the surface of the capsules [Figure 2b (panel 2)] and also packed within the interior

of capsules [Figure 2b (panel 3)]. Furthermore, it was interesting to note that short-chain lactobacilli were observed inside the yeast capsules [Figure 2b (panel 3)] and these bacteria were surrounded by yeast cells. According to the scanning electron microscopy photos, cell growth at the beads destroyed gel structure, as also observed by Gosmann and Rehm (1988) and Audet et al. (1991). Thus, lactobacilli might find a way to the center of yeast beads. Additionally, this close proximity presumably indicates some sort of physical-chemical

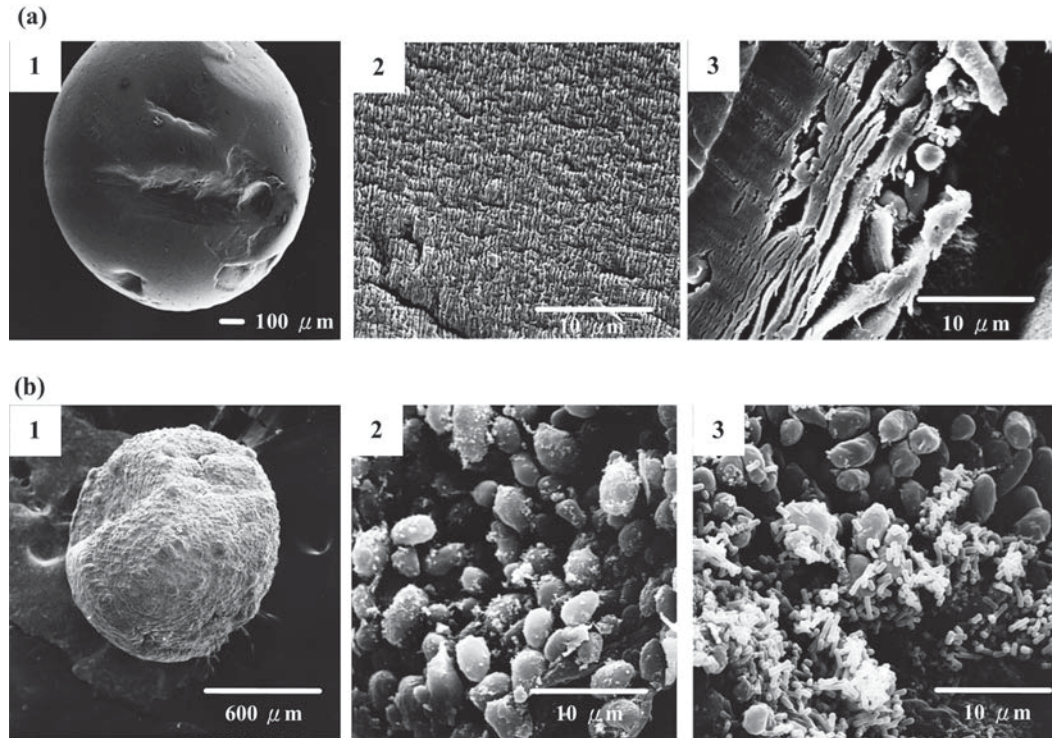


Figure 2. Scanning electron micrograph showing the effect of number of fermentation cycles on the entrapped yeast: (a) 0 cycles; (b) 28 cycles. Panel 1 = whole gel bead; 2 = bead surface; 3 = bead cross-section.

interaction. Farnworth and Mainville (2003) reported that the yeasts found in kefir grains were essential to maintaining the integrity and viability of the microflora population. Amino acids and other essential growth factors for the bacteria are produced by yeasts, whereas bacterial metabolic end-products are used as energy sources by the yeasts. The symbiosis found in the kefir grain microflora allows the grains to maintain uniformity so that, throughout the year, the microbiological profile of kefir grains and kefir remains stable.

The microbiological composition of the kefir grains (Figure 3) consists of a high density of yeast, lactococci, and short-chain lactobacilli on the surface [Figure 3a (panel 2)] and long-chain and short-chain LAB in the core [Figure 3a (panel 3)]. No morphological difference was found from the first to the last fermentation cycles. The microbiological population appears to be different on the surface compared with the interior of both types of microspheres and the kefir grains (Figures 1, 2, and 3). This might be due, in part, to pH differences in different parts of the microspheres and grains; specifically, the interior of grains has been reported to have a very low pH that inhibits the growth of lactococci (Rea et al., 1996; Witthuhn et al., 2004). Lacroix et al. (2005) also reported that conditions are more favorable for certain cell growth close to the bead surface because of

diffusion limitations for both substrates and inhibitory products, mainly lactic acid in the case of LAB, which results in a sharp pH gradient in beads.

Microbiological Properties of Kefir Produced by Entrapped Starter Cultures

An increase in the cell counts with the number of fermentation cycles for kefir from microspheres (microcapsule kefir) and from grains (traditional kefir), as shown in Figure 4, was observed. Immediately after the addition of microspheres to the milk, LAB reached a concentration of 10^4 cfu/mL and yeasts reached 10^3 cfu/mL. This indicated that part of the microflora contained in the capsule was immediately transferred to milk. Lacroix et al. (2005) explained that cell release from gel beads in the liquid medium occurs spontaneously because of the formation of the high-biomass-density peripheral layer at the bead surface. The use of entrapped kefir cultures as the initial inoculum resulted in a lag period of up to 4 fermentation cycles before the filtrate reached a normal cell population for kefir. Similarly, the use of inactive kefir grains (in this case specifically frozen grains) resulted in a lag period of up to 2 fermentation cycles (Figure 4). This indicates that it is important to activate the entrapped starter

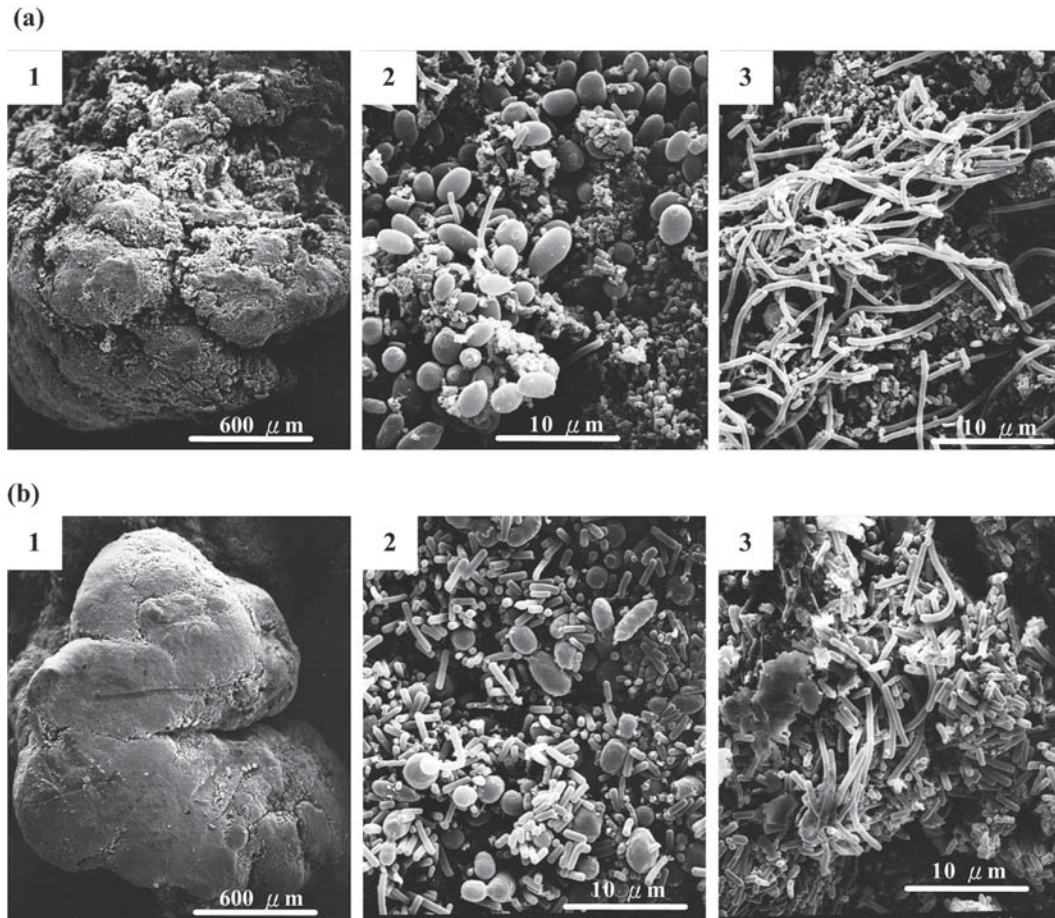


Figure 3. Scanning electron micrograph showing the effect of number of fermentation cycles on kefir grains: (a) 0 cycles; (b) 28 cycles. Panel 1 = whole gel bead; 2 = bead surface; 3 = bead cross-section.

cultures as well as kefir grains before using them for cultivation. It is worth noting that the kefir made from microspheres showed lower LAB and yeast population levels during early fermentation cycles. Perhaps only a few of the LAB and yeasts bound in the alginate beads were released from the bead surface to the bulk medium during the early fermentation cycles because of the low-biomass-density peripheral layer at the bead surface. The microscopy results on the microspheres (Figures 1 and 2) supported this hypothesis, with few or no microflora present on the surface of the capsules during the early fermentations; then, after several fermentation cycles, dense cells could be observed. Audet et al. (1991) also reported that peripheral gel cavities containing microcolonies are disrupted by cell growth and shear forces during continuous culture of immobilized LAB, resulting in acceleration of cell release.

The distribution of the species of LAB and yeasts in the kefir from the grains and from the microspheres is given in Table 1. *Leuconostoc mesenteroides* and *K. marxianus* were the predominant microflora in both

kefir. There was no difference among fermentation cycles. In our previous study (Wang et al., 2008), *Klu. marxianus*, a lactose- and galactose-positive yeast, accounted for 76% of total isolates from kefir grains and it is also the most dominant yeast found in these kefir grains. *Lactobacillus kefir* accounted for 53% of the total LAB isolates, making it the most dominant LAB found in the kefir grains. This species was followed by *Lb. kefiranofaciens* (43%) and *Leu. mesenteroides* (4%). Nonetheless, *Leu. mesenteroides* was found to be the predominant LAB species in the microflora of both types of kefir. Several studies (Garrote et al., 1998; Beshkova et al., 2002) have revealed that lactococci are the most numerous LAB in the final kefir product. The microorganism profile of the final product does not necessarily parallel that of the grains because of the conditions during the fermentation process. In addition, the location of the microorganisms in the grains may be a factor (Farnworth and Mainville, 2003). Lactococci are found on the periphery of the capsules (Figure 1), whereas the lactobacilli are present inside the alginate

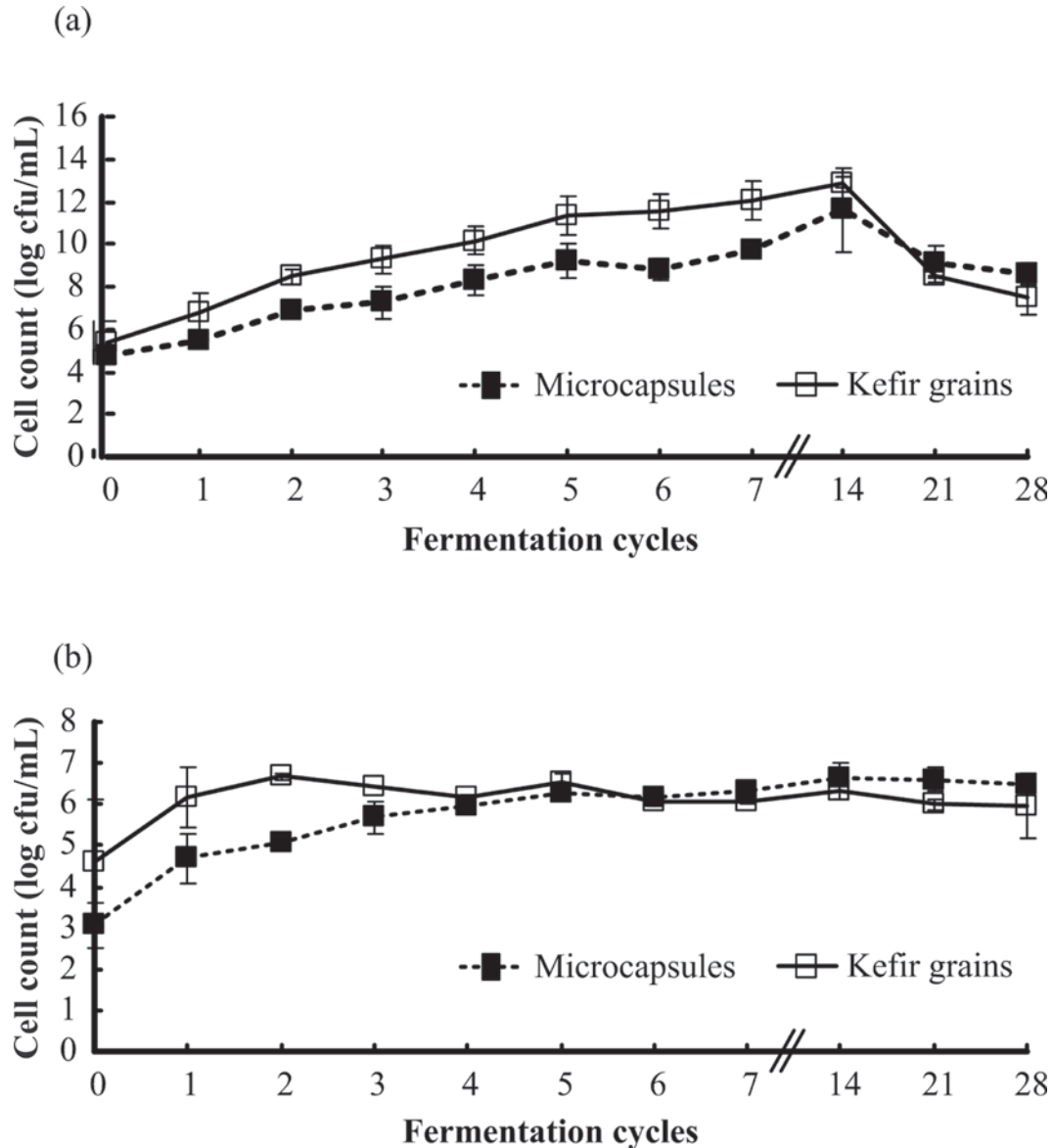


Figure 4. Effect of number of fermentation cycles on the cell counts of lactic acid bacteria (a) and yeast (b) in kefir produced by entrapped kefir starter culture (microcapsules) or kefir grain.

beads. Thus, it is likely that the number of lactococci immediately released into the milk is higher than that of other LAB.

Each strain provides a unique characteristic for kefir. As noted by Koroleva (1991), the growth and survival of individual strains are dependent on the presence of others. Toba (1987) indicated several possible metabolic products that might contribute to the symbiotic relationship in kefir grains. The growth of *Lb. kefir* was enhanced when *Candida kefir* was added, either before or simultaneously, to the milk to be fermented (Linossier and Dousset, 1994). Growth of the yeast, however, was not stimulated by the presence of the bacterium. When

the 2 organisms were cultured together, the amounts of lactic acid, glycerol, and ethanol produced were increased (Farnworth and Mainville, 2003).

Chemical Properties of Kefir Produced by Entrapped Culture Starters

For kefir made from grains, the pH dropped sharply from 6.7 to <4.3 during the first fermentation cycles and then slowly continued to decrease to pH 3.5 over the duration of the study (Table 2), even though the milk was removed daily and replaced with fresh milk. A strong increase in titratable acid (TA) was also found

Table 1. The effect of number of fermentation cycles on the distribution frequency (%) of lactic acid bacteria and yeast in kefir produced by entrapped kefir starter culture or kefir grain

Distribution (%)	Kefir type ¹	Number of fermentation cycles			
		7	14	21	28
Lactic acid bacteria					
<i>Lactobacillus kefirnofaciens</i>	M	2.8 ± 0.1 ^{a,x}	3.8 ± 1.9 ^{a,x}	3.5 ± 1.3 ^{a,x}	2.9 ± 0.1 ^{a,x}
	K	1.5 ± 1.2 ^{a,x}	1.3 ± 0.8 ^{a,x}	7.0 ± 0.5 ^{c,y}	3.1 ± 0.2 ^{b,x}
<i>Lactobacillus kefir</i>	M	5.6 ± 0.2 ^{a,y}	3.8 ± 1.9 ^{a,x}	3.5 ± 1.3 ^{a,x}	5.9 ± 0.2 ^{a,y}
	K	1.5 ± 1.1 ^{a,x}	4.0 ± 1.8 ^{a,x}	3.5 ± 1.4 ^{a,x}	3.1 ± 0.2 ^{a,x}
<i>Leuconostoc mesenteroides</i>	M	91.6 ± 3.6 ^{a,x}	92.4 ± 3.3 ^{a,x}	93.0 ± 5.2 ^{a,x}	91.2 ± 3.7 ^{a,x}
	K	97.0 ± 0.2 ^{a,y}	94.7 ± 0.1 ^{a,x}	89.5 ± 3.4 ^{a,x}	93.8 ± 4.0 ^{a,x}
Yeast					
<i>Kluyveromyces marxianus</i>	M	94.4 ± 2.6 ^{a,x}	93.0 ± 0.5 ^{a,x}	88.6 ± 4.0 ^{a,x}	94.5 ± 0.1 ^{a,y}
	K	97.2 ± 0.2 ^{a,x}	95.2 ± 2.1 ^{a,x}	95.2 ± 2.3 ^{a,y}	88.2 ± 5.6 ^{a,x}
<i>Saccharomyces turicensis</i>	M	1.9 ± 1.3 ^{a,x}	3.5 ± 0.3 ^{b,x}	8.6 ± 0.1 ^{c,y}	1.4 ± 1.0 ^{a,x}
	K	1.4 ± 1.2 ^{a,x}	3.2 ± 0.1 ^{b,x}	1.6 ± 1.3 ^{a,x}	5.9 ± 0.2 ^{c,y}
<i>Pichia fermentans</i>	M	3.7 ± 0.1 ^{a,y}	3.5 ± 0.2 ^{a,y}	2.9 ± 0.1 ^{a,x}	8.1 ± 1.8 ^{b,y}
	K	1.4 ± 1.0 ^{a,x}	1.6 ± 1.1 ^{a,x}	4.8 ± 2.1 ^{b,x}	5.9 ± 0.2 ^{b,x}

^{a-c}Values in the same row bearing different letters are significantly different ($P < 0.05$).

^{x,y}Values in the same column and species bearing different letters are significantly different ($P < 0.05$).

¹M = kefir produced by entrapped kefir starter culture; K = kefir produced by kefir grains.

in the kefir made from grains (Table 2). After an initial major increase, the TA values stabilized between 1.28 and 1.45% and did not show any major changes over the remaining fermentation cycles. In contrast, the pH of the kefir produced from microspheres dropped slowly over the first 7 batch fermentation cycles to reach a pH value below 5.0 and then the pH stabilized in the 4.4 to 4.6 range. The TA result generally paralleled the pH, reaching an average value of 0.6% after 4 batch fermentation cycles and then slowly increasing to 1.0% progressively over the duration of the study. The measured TA is generally considered to parallel the amount of lactic acid produced within the kefir (Table 2). The lactic acid content of kefir made from grains reached 6.4 g/L after 3 fermentation cycles and continued to increase progressively, reaching 7.5 g/L by the end of the study. It is noteworthy that kefir made from grains had a higher TA concentration, a higher lactic acid concentration, and a lower pH than the kefir made from microspheres. The difference between the traditional kefir and microcapsule kefir may be a result of the differences in microbiological profile between the 2 inocula or symbiosis within the microorganism population of the kefir grains, which is probably more limited in the capsules. Although symbiosis was present in the entrapped kefir starter cultures, the fact that the LAB and yeast were entrapped in different microspheres might have interfered with any interaction between the LAB and yeasts. In addition, the effective diffusion coefficients for lactose and lactic acid were reduced proportionally to the bacterial concentration in the alginate gel beads (Øyaas et al., 1995). The effective diffusion coefficients were significantly lower at pH 4.5 than at pH 5.5 and

6.5 for both lactose and lactic acid. The above factors might be the reasons for the higher pH in the kefir made from entrapped cultures.

The ethanol content and productivity (Table 2) increased slightly but significantly ($P < 0.05$) during fermentation cycles, reaching a final value of 0.7% and 0.3 g/L per hour, respectively, after 7 fermentation cycles for both types of inoculum. This value is within the range of values observed by other authors for kefir from different origins. However, the production of ethanol was significantly lower in the entrapped kefir cultures compared with grains before 6 fermentation cycles. This result was supported by the development of the yeast population in kefir. In the traditional kefir, the viable yeast population count was 10^6 cfu/mL during batch fermentation cycles 1 to 5 compared with 10^4 to 10^5 cfu/mL for the microcapsule kefir. Other research (Beshkova et al., 2002; Farnworth and Mainville, 2003) has indicated that the final alcohol concentration in kefir is determined, for the most part, by the amount of yeast present in the grains that are added to the milk and on the duration of fermentation.

In addition, immobilization of kefir yeast cells for continuous production of fermented beverage, potable alcohol, and biofuel alcohol from whey as substrate has received considerable attention in recent years (Kourkoutas et al., 2002; Dimitrellou et al., 2008). Our ethanol productivity values (Table 2) are within the range of values observed by other authors for production of alcohol using thermal-dried kefir biomass (Papapastolou et al., 2008) or immobilized kefir yeasts (Kourkoutas et al., 2002) as starter cultures. This technique might have potential to produce alcohol using whey as a sub-

Table 2. Effects of number of fermentation cycles on chemical properties in the kefir produced by entrapped kefir starter culture or kefir grain¹

Cycles	pH value		Titratable acid (%)		Lactic acid (g/L)		Ethanol (% vol/vol)		EPS ² (µg/mL)		Lactic acid productivity (g/L per hour)		Ethanol productivity (g/L per hour)	
	M	K	M	K	M	K	M	K	M	K	M	K	M	K
0	6.45 ^{ax}	6.45 ^{ax}	0.25 ^{ax}	0.25 ^{ax}	3.25 ^{bx}	3.23 ^{bx}	0.00 ^{bx}	0.00 ^{bx}	0.00 ^{ax}	0.00 ^{ax}	0.14 ^{ax}	0.13 ^{ax}	0.00 ^{ax}	0.00 ^{bx}
1	5.78 ^{bx}	4.34 ^{by}	0.34 ^{bx}	0.94 ^{by}	3.44 ^{bx}	4.79 ^{by}	0.00 ^{ax}	0.05 ^{ax}	18.23 ^{bx}	35.87 ^{by}	0.14 ^{ax}	0.20 ^{by}	0.00 ^{ax}	0.02 ^{by}
2	5.33 ^{cx}	4.17 ^{by}	0.46 ^{cx}	0.92 ^{by}	4.94 ^{cx}	5.52 ^{by}	0.06 ^{bx}	0.43 ^{by}	25.29 ^{bcx}	62.92 ^{cy}	0.21 ^{bx}	0.23 ^{cy}	0.03 ^{bx}	0.18 ^{cy}
3	5.38 ^{dx}	4.19 ^{by}	0.46 ^{cx}	0.91 ^{by}	5.27 ^{dx}	6.43 ^{by}	0.07 ^{bx}	0.44 ^{by}	24.70 ^{bcx}	81.15 ^{dy}	0.22 ^{bx}	0.27 ^{dy}	0.03 ^{bx}	0.18 ^{cy}
4	5.23 ^{ex}	4.07 ^{cy}	0.67 ^{dx}	1.02 ^{cy}	5.51 ^{ex}	6.71 ^{cy}	0.11 ^{bx}	0.51 ^{by}	29.99 ^{cx}	95.26 ^{ey}	0.23 ^{cx}	0.28 ^{dey}	0.05 ^{cx}	0.21 ^{dy}
5	5.15 ^{fx}	4.05 ^{cy}	0.68 ^{dx}	1.05 ^{cy}	5.71 ^{fx}	6.49 ^{by}	0.29 ^{cx}	0.50 ^{by}	65.86 ^{dx}	94.67 ^{ey}	0.24 ^{cx}	0.27 ^{dy}	0.12 ^{dx}	0.21 ^{dy}
6	5.03 ^{gx}	4.03 ^{cy}	0.62 ^{dx}	1.19 ^{dy}	6.31 ^{gx}	6.74 ^{cy}	0.41 ^{dx}	0.72 ^{cy}	83.50 ^{ex}	92.32 ^{fy}	0.26 ^{dx}	0.29 ^{ey}	0.17 ^{cx}	0.30 ^{fy}
7	4.94 ^{hx}	3.95 ^{dy}	0.71 ^{dx}	1.29 ^{dey}	6.43 ^{hx}	7.01 ^{fy}	0.63 ^{cx}	0.67 ^{cx}	93.50 ^{fx}	124.07 ^{fy}	0.27 ^{dx}	0.29 ^{ey}	0.26 ^{fx}	0.28 ^{ex}
14	4.75 ^{ix}	3.78 ^{ey}	0.74 ^{dx}	1.35 ^{ey}	6.84 ^{ix}	7.20 ^{gy}	0.73 ^{fx}	0.72 ^{cx}	126.41 ^{gx}	123.48 ^{fx}	0.29 ^{cx}	0.30 ^{ex}	0.30 ^{gx}	0.30 ^{fx}
21	4.54 ^{ix}	3.54 ^{fy}	0.84 ^{ex}	1.45 ^{fy}	6.66 ^{ix}	7.26 ^{gy}	0.68 ^{cx}	0.71 ^{cx}	116.43 ^g	119.37 ^{fx}	0.28 ^{ex}	0.30 ^{ex}	0.28 ^{gx}	0.30 ^{ex}
28	4.50 ^{ix}	3.41 ^{gy}	0.95 ^{fx}	1.50 ^{fy}	7.22 ^{ix}	7.56 ^{hy}	0.65 ^{bx}	0.67 ^{cx}	115.23 ^g	118.19 ^{fx}	0.30 ^{fx}	0.32 ^{fx}	0.27 ^{gx}	0.28 ^{fx}

^{a-k}Values in the same column bearing different letters are significantly different ($P < 0.05$).

^{x-y}Values in the same row within the same chemical characteristic bearing different letters are significantly different ($P < 0.05$).

¹M = kefir produced by entrapped kefir starter culture; K = kefir produced by kefir grains.

²EPS = exopolysaccharide.

strate. Several studies suggested that the use of whey in mixtures with low-cost raw materials such as molasses or raisin extracts and the application of a continuous process may significantly increase ethanol productivity and decrease alcohol production costs (Kourkoutas et al., 2002; Koutinas et al., 2007).

The formation of EPS increased slightly during cultivation, reaching a final value of 120 µg/mL by 7 fermentation cycles for both samples. The EPS content of the microcapsule kefir was lower than that of traditional kefir at 7 fermentation cycles; after this, both kefir have the same EPS content and the amount remains stable. The bacteria that produce EPS have been the subject of additional research. *Lactobacillus kefirianofaciens*, which was present in our microspheres and grains, is known to be a polysaccharide-producing bacterium (Toba et al., 1991). Although EPS is found in kefir grains, few reports have been published on the concentration of EPS in the final drink (Koroleva, 1991; Farnworth and Mainville, 2003). Nonetheless, EPS may contribute significantly to the texture of kefir.

CONCLUSIONS

Kefir quality depends on various properties such as chemical composition, microflora, and rheology features. By using entrapped starter cultures that had been isolated from kefir grains, the basic microbiological and physicochemical characteristics of traditional kefir were reproduced. This alternative production procedure for kefir eliminates the problems that arise from the use of kefir grains and should make possible the production of a standard product by the dairy industry. However, the use of entrapped kefir cultures as initial inoculum did result in a lag period before the filtrate reached the normal cell population for a kefir and therefore it is important to activate the entrapped starter cultures before using them for making kefir.

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REFERENCES

Arnaud, J. P., C. Lacroix, and L. Choplin. 1992. Effect of agitation rate on cell release rate and metabolism during continuous fermentation with entrapped growing *Lactobacillus casei* subsp. *casei*. *Biotechnol. Tech.* 6:265-270.

Assadi, M. M., F. Abdolmaleki, and R. R. Mokarrame. 2008. Application of whey in fermented beverage production using kefir starter culture. *Nutr. Food Sci.* 38:121-127.

Assadi, M. M., R. Pourahmad, and N. Moazami. 2000. Use of isolated kefir starter cultures in kefir production. *World J. Microbiol. Biotechnol.* 16:541-543.

Athanasiadis, I., D. Boskou, M. Kanellaki, V. Kiosseoglou, and A. A. Koutinas. 2002. Whey liquid waste of the dairy industry as raw

- material for potable alcohol production by kefir granules. *J. Agric. Food Chem.* 50:7231–7234.
- Athanasiadis, I., A. Paraskevopoulou, G. Blekas, and V. Kiosseoglou. 2004. Development of a novel whey beverage by fermentation with kefir granules. Effect of various treatments. *Biotechnol. Prog.* 20:1091–1095.
- Audet, P., C. Lacroix, and C. Paquin. 1991. Continuous fermentation of a whey supplemented whey permeate medium with immobilized *Streptococcus salivarius* subsp. *thermophilus*. *Int. Dairy J.* 1:1–15.
- Bergmaier, D., C. P. Champagne, and C. Lacroix. 2005. Growth and exopolysaccharide production during free and immobilized cell chemostat culture of *Lactobacillus rhamnosus* RW-9595M. *J. Appl. Microbiol.* 96:272–284.
- Beshkova, D. M., E. D. Simova, Z. I. Simov, G. I. Frengova, and Z. N. Spasov. 2002. Pure cultures for making kefir. *Food Microbiol.* 19:537–544.
- Cachon, R., P. Antérieux, and C. Diviès. 1998. The comparative behavior of *Lactococcus lactis* in free and immobilized culture processes. *J. Biotechnol.* 63:211–218.
- Cerning, J., C. M. G. C. Renard, J. F. Thibault, C. Bouillanne, M. Landon, and M. Desmazeaud. 1994. Carbon source requirements for exopolysaccharide production by *Lactobacillus casei* CG11 and partial structure analysis of the polymer. *Appl. Environ. Microbiol.* 60:3914–3919.
- Chai, Y., L. Mei, G. Wu, D. Lin, and S. Yao. 2004. Gelation conditions and transport properties of hollow calcium alginate capsules. *Biotechnol. Bioeng.* 87:228–233.
- Chen, H. C., S. Y. Wang, and M. J. Chen. 2008. Microbiological study of lactic acid bacteria in kefir grains by culture-dependent and culture-independent methods. *Food Microbiol.* 25:492–502.
- Chen, M. J., K. N. Chen, and Y. T. Kuo. 2007. Optimal thermotolerance of *Bifidobacterium bifidum* in gellan-alginate microparticles. *Biotechnol. Bioeng.* 98:411–419.
- Chen, M. J., J. R. Liu, J. F. Sheu, C. W. Lin, and C. L. Chuang. 2006. Study on skin care properties of milk kefir whey. *Asian Australas. J. Anim. Sci.* 19:905–908.
- Desmond, C., R. P. Ross, E. O'Callaghan, G. Fitzgerald, and C. Stanton. 2002. Improved survival of *Lactobacillus paracasei* NFBC 338 in spray-dried powders containing gum acacia. *J. Appl. Microbiol.* 93:1003–1011.
- Dimitrellou, D., Y. Kourkoutas, I. M. Banat, R. Marchant, and A. A. Koutinas. 2007. Whey cheese production using freeze-drying kefir co-culture as a starter. *J. Appl. Microbiol.* 103:1170–1183.
- Dimitrellou, D., K. Tsaousi, Y. Kourkoutas, P. Panas, M. Kanellaki, and A. A. Koutinas. 2008. Fermentation efficiency of thermally dried immobilized kefir on casein as starter culture. *Process Biochem.* 43:1323–1329.
- Duitschaever, C. L., N. Kemp, and E. Emmons. 1987. Pure culture formulation and procedure for the production of kefir. *Milchwissenschaft* 42:80–82.
- Duitschaever, C. L., N. Kemp, and E. Emmons. 1988. Comparative evaluation of five procedures for making kefir. *Milchwissenschaft* 43:343–345.
- Farnworth, E. R., and I. Mainville. 2003. Kefir: A fermented milk product. Pages 77–112 in *Handbook of Fermented Functional Foods*. E. R. Farnworth, ed. CRC Press, London, UK.
- Garrote, G. L., A. G. Abraham, and G. L. Antoni. 1998. Characteristics of kefir prepared with different grain:milk ratios. *J. Dairy Res.* 65:149–154.
- Goh, K. K. T., D. R. Haisman, R. H. Archer, and H. Singh. 2005. Evaluation and modification of existing methods for the quantification of exopolysaccharides in milk-based media. *Asian Australas. J. Anim. Sci.* 38:605–613.
- Göksungur, Y., and N. Zorlu. 2001. Production of ethanol from beet molasses by ca-alginate immobilized yeast cells in a packed-bed bioreactor. *Turk. J. Biol.* 25:265–275.
- Gosmann, B., and H. Rehm. 1988. Influence of growth behavior and physiology of alginate-entrapped microorganisms on the oxygen consumption. *Appl. Microbiol. Biotechnol.* 29:554–559.
- Harrigan, W. F. 1998. *Laboratory Methods in Food Microbiology*. Pages 89–91. Academic Press, San Diego, CA.
- Jan, G., P. Leverrier, B. Pichereau, and V. Boyaval. 2001. Changes in protein synthesis and morphology during acid adaptation of *Propionibacterium freudenreichii*. *Appl. Environ. Microbiol.* 67:2029–2036.
- Koroleva, N. S. 1991. Products prepared with lactic acid bacteria and yeasts. Pages 159–179 in *Therapeutic Properties of Fermented Milk*. R. K. Robinson, ed. Elsevier Applied Science, London, UK.
- Kourkoutas, Y., P. Kandylis, P. Panas, J. S. G. Dooley, P. Nigam, and A. A. Koutinas. 2006. Evaluation of freeze-drying kefir co-culture as a starter in Feta-type cheese production. *Appl. Environ. Microbiol.* 72:6124–6135.
- Kourkoutas, Y., C. Psarianos, A. A. Koutinas, M. Kanellaki, I. M. Banat, and R. Marchant. 2002. Continuous whey fermentation using kefir yeast immobilized on delignified cellulose material. *Agric. Food Chem.* 50:2543–2547.
- Koutinas, A. A., I. Athanasiadis, A. Bekatorou, C. Psarianos, M. Kanellaki, N. Agouridis, and G. Blekas. 2007. Kefir-yeast technology: Industrial scale-up of alcoholic fermentation of whey, promoted by raisin extracts, using kefir-yeast granular biomass. *Enzyme Microb. Technol.* 41:576–582.
- Lacroix, C., F. Grattepanche, Y. Doleys, and D. Bergmaier. 2005. Immobilised cell technologies for the dairy industry. Pages 295–319 in *Applications of cell immobilisation biotechnology*. V. Nedović and R. Willaert, ed. Springer, Dordrecht, the Netherlands.
- Libudzisz, Z., and A. Piatkiewicz. 1990. Kefir production in Poland. *Dairy Ind. Int.* 55:31–33.
- Lin, C. W., H. L. Chen, and J. R. Liu. 1999. Identification and characterisation of lactic acid bacteria and yeasts isolated from kefir grains in Taiwan. *Aust. J. Dairy Technol.* 54:14–18.
- Lin, T. Y., T. H. Hung, and T. S. Cheng. 2005. Conjugated linoleic acid production by immobilized cells of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Lactobacillus acidophilus*. *Food Chem.* 92:23–28.
- Linossier, J. P., and X. Dousset. 1994. Stimulation de la croissance et du métabolisme de *Lactobacillus kefir* par *Candida kefir*. *Microbiol. Aliments Nutr.* 12:341–351.
- Liu, J. A. P., and N. J. Moon. 1983. Kefir—A new fermented milk product. *Cult. Dairy Prod. J.* 18:11–12.
- Marshall, V. M., and W. M. Cole. 1985. Methods for making kefir and fermented milks based on kefir. *J. Dairy Res.* 52:451–456.
- Masson, F., C. Lacroix, and C. Paquin. 1994. Direct measurement of pH profiles in gel beads immobilizing *Lactobacillus helveticus* using a pH sensitive microelectrode. *Biotechnol. Tech.* 8:551–556.
- Motaghi, M., M. Mazaheri, N. Moazami, A. Farkhondeh, M. H. Fooladi, and E. M. Goltapeh. 1997. Short communication: Kefir production in Iran. *World J. Microbiol. Biotechnol.* 13:579–581.
- Nagashima, M., M. Azuma, S. Noguchi, K. Inuzuka, and H. Samejima. 1984. Continuous ethanol fermentation using immobilized yeast cells. *Biotechnol. Bioeng.* 36:992–997.
- Øyaas, J., I. Storrø, H. Svendsen, and D. W. Levine. 1995. The effective diffusion coefficient and the distribution constant for small molecules in calcium-alginate gel beads. *Biotechnol. Bioeng.* 47:492–500.
- Papapastolou, H., L. A. Bosnea, A. A. Koutinas, and M. Kanellaki. 2008. Fermentation efficiency of thermally dried kefir. *Bioresour. Technol.* 99:6949–6956.
- Plessas, S., L. Pherson, A. Bekatorou, P. Nigam, and A. A. Koutinas. 2005. Bread making using kefir grains as baker's yeast. *Food Chem.* 93:585–589.
- Rea, M. C., T. Lennartsson, P. Dillon, F. D. Drinan, W. J. Revilli, M. Heapes, and T. M. Cogan. 1996. Irish kefir-like grains: Their structure, microbial composition and fermentation kinetics. *J. Appl. Bacteriol.* 81:83–94.
- Schoevers, A., and T. J. Britz. 2003. Influence of different culturing conditions on kefir grain increase. *Int. J. Dairy Technol.* 56:183–187.
- Sheu, T. Y., and R. T. Marshall. 1993. Microentrapment of lactobacilli in calcium alginate gels. *J. Food Sci.* 54:557–561.

- Skjak-Brak, G., K. H. Grasdale, K. I. Draget, and O. Smidsrod. 1989. In homogeneous polysaccharide ionic gels. *Carbohydr. Polym.* 10:31–54.
- Suwannakham, S., and S. Yang. 2005. Enhanced propionic acid fermentation by *Propionibacterium acidipropionici* mutant obtained by adaptation in a fibrous-bed bioreactor. *Biotechnol. Bioeng.* 91:325–337.
- Toba, T. 1987. Fermented milks produced with mesophilic lactic acid bacteria: Kefir and Scandinavian rosy sour milk. *Jpn. J. Dairy Food Sci.* 36:2–4.
- Toba, T., H. Uemura, T. Mukai, Y. Fujii, T. Itoh, and S. Adachi. 1991. A new fermented milk using capsular polysaccharide-producing *Lactobacillus kefiranoferiens* isolated from kefir grains. *J. Dairy Res.* 58:497–502.
- Wang, S. Y., H. C. Chen, J. R. Liu, Y. C. Lin, and M. J. Chen. 2008. Identification of yeasts and evaluation of their distribution in Taiwanese kefir and viili starters. *J. Dairy Sci.* 91:3798–3805.
- Witthuhn, R. C., T. Schoeman, and T. J. Britz. 2004. Isolation and characterization of the microbial population of different South African kefir grains. *Int. J. Dairy Technol.* 57:33–37.
- Ye, K., M. Shijo, K. Miyano, and K. Shimizu. 1999. Metabolic pathway of *Propionibacterium* growing with oxygen: Enzyme, ¹³C NMR analysis, and its application for vitamin B12 production with periodic fermentation. *Biotechnol. Prog.* 15:201–207.