Hindawi
Journal of Food Quality
Volume 2020, Article ID 8839135, 8 pages
https://doi.org/10.1155/2020/8839135

Research Article

Prospects of Artificial Kefir Grains Prepared by Cheese and Encapsulated Vectors to Mimic Natural Kefir Grains

Xue Han,[1] **Huaxi Yi**,[2] **Sainan Zhao**,[1] **Jialei Sun**,[1] **and Yu Wang**[1]

[1]*School of Chemistry and Chemical Engineering, Harbin Institute of Technology, Harbin, Heilongjiang 150001, China*
[2]*College of Food Science and Engineering, Ocean University of China, Qingdao 266003, China*

Correspondence should be addressed to Xue Han; xhan@hit.edu.cn

Received 24 June 2020; Revised 15 September 2020; Accepted 23 September 2020; Published 5 October 2020

Academic Editor: Fatma M. El-Demerdash

Kefir is a natural fermented dairy beverage obtained by fermenting milk with kefir starter grains. However, up to now, there is still no efficient approach to producing stable kefir grains by using the pure or cultural mixture of strains isolated from the original kefir grains. Therefore, new techniques need to be taken to promote the kefir grain production. To this purpose, an encapsulated vector produced by entrapment of the dominant strains isolated from kefir grain and the cheese vector which was produced by a traditional manufacturing method was used to mimic kefir grain forming, respectively. Then, the composition, microstructure, and microflora of the two vectors were investigated and were compared with the natural kefir grains. Results indicated that the protein and polysaccharide content of cheese vector were much higher than encapsulated vector; the distribution of microorganisms inside the cheese vector was more similar to that inside the natural kefirs. It indicated that the cheese vector would be more suitable to mimic kefir grain production. Results of the present investigations reveal the potential of the cheese vector for kefir grains production at the industrial level.

1. Introduction

Kefir is a viscous, acidic, and slightly carbonated fermented dairy product known from ancient times, which became very popular recently. [1, 2]. Kefir became a kind of popular natural fermented probiotic drink not only for its nutritional value but also for its effectiveness [3], such as antimicrobial activity [4] and antiallergenic [5], antimutagenic [6], antitumoral [7], and hypocholesterolemic [8] effects, against a variety of complaints and diseases. Since kefir has been consumed by people for centuries, it is considered safe for human health and is classified by the US Food and Drug Administration (FDA) as "generally considered as safe (GRAS)" [9].

Traditionally, kefir fermentation starts with an addition of "kefir grains" to mammalian milk. Kefir grains consist of a combination of lactic acid bacteria (*Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *Streptococcus spp.*), acetic acid bacteria (*Acetobacter*), and yeasts (*Kluyveromyces*, *Saccharomyces*, and *Torula*), which are held together by a matrix of complex sugars composed of a mixture of polysaccharide and casein in semihard granules [1]. Now, more than 50 microbial species are identified in different milk kefirs [10, 11]. The microbial composition of kefir grains can change due to several factors, such as the cultivation conditions (e.g., temperature, grain to milk ratio, and milk source) and the geographical origin of kefir [4]. The origins of kefir grains varied, ranging from that in Argentina, Brazil, Belgium, China, Ireland, Malaysia, Russia, and Turkey [12]. Therefore, the main factors that influence the development of kefir industry are the source of kefir grains and its cultivation method.

Kefir grains are initially very small, but they increase in size during fermentation and they can only grow from the preexisting grain. Traditionally, the growth of kefir grains is achieved by continuous passage of kefir grains into the milk, resulting in biomass increases of 5–7% per day [13]. However the biomass increase of kefir grains by traditional methods is not stable and growth very slow.

Recently, various researches have been undertaken to improve the kefir grain production such as grain : milk ratio, cultivation temperature, period of time, and conditions prior to separation of grains from the fermented milk, washing of kefir grains, and so on. All these factors influence the microflora of the kefir starter and fermented milk. The complex microbiological composition of kefir grains explains why it is difficult to obtain a starter with the optimal and constant composition necessary for a regular kefir production of standard quality [13, 14]. Several studies on making kefir grains without preexisting grains have been carried out [15, 16]. But until now, no successful grains were formed.

Apparently, it is difficult to ferment stable kefir without stable kefir grains [17]. Furthermore, there is still no efficient approach to producing stable kefir grains by using the pure or cultural mixture of strains isolated from the original grains [18]. New techniques need to be taken to promote the kefir grains production. Hence, the objective of this study was to use the cheese and encapsulated vector to produce kefir grains artificially. Comparative studies on chemical properties, microstructure, and microflora of different vectors and natural grains were also undertaken.

2. Materials and Methods

2.1. Kefir Grains and Culture Conditions. Kefir grains, obtained from a household in Harbin, China, were activated using pasteurized cow milk at 25°C followed by the removal of the clotted milk by filtering with a sieve and rinsing with sterile water. Finally, these strains were stored at 4°C [19].

2.2. Cheese Vector Preparation. The cheese vector was produced by a traditional manufacturing method [20]. 12% skimmed milk was inoculated with 5% (w/w) kefir milk which was fermented by 2% (w/v) kefir grains at 28°C for 24 h. When the pH of fermented milk reached 5.8 at 37°C, 0.002% (w/v) rennet (Hannilase L, 690 IMCU/mL, Chr. Hansen) and 0.01% (w/v) $CaCl_2$ were added and stirred. The fermentation was not finished until coagulation was achieved. The resulting cheese curd was cut into 1 cm^3 cubes and submitted to slow continuous mixing for 5 min. After that, the sweet whey was drained off. Finally, the curd was pressed at 600 kPa for 1 h and then stored at 4°C.

2.3. Encapsulated Vector Preparation. Kefir grains were cultured in De Man Rogosa Sharpe (MRS) Broth and M17 Broth at 37°Cfor 48 h and Potato Dextrose Agar (PDA) broth (OXOID Ltd., China) at 28°Cfor 24 h, respectively. The fermented products of kefir grains were harvested by centrifuging at 3000×g for 10 min and then suspended in 2.0% sterilized sodium alginate solution. The alginate droplets containing bacterial cells were injected into 0.1 $mol \cdot L^{-1}$ $CaCl_2$ solution through a 22-gauge sharp-pointed injection needle (outer diameter, 0.7 mm). After being blended and stirred for 1 h, the droplets became hard gradually and formed capsule particle with a diameter of about 5–8 mm and then stored in 0.1% phosphate buffer at 4°C.

2.4. Chemical Analysis. Samples were rinsed with sterile water and blotted by filter paper. Moisture was determined by the oven-drying method at 103 ± 2°C [21]. The nitrogen content was determined by the Kjeldahl method and the final crude protein content was multiplied by a factor of 6.38 [22]. Polysaccharide was determined by phenol-sulphate acid method [23]. All of the analyses were performed in triplicate.

2.5. Physical Structure Analysis. Samples were cut into 1 mm^3 cubic pieces and immersed in 2.5% glutaraldehyde fixative for 4 h and then washed by 0.1 mol/L phosphate buffer (pH 6.8, 25°C) for three times. After being dehydrated in a graded ethanol series (50%, 70%, and 90%) and defatted in a 1 : 1 ratio of chloroform/acetone, the fragments were freeze-fractured in liquid nitrogen and then mounted on aluminum stubs by silver paint and coated with gold for 6 min in a sputter coater. At least four images of typical structures were recorded using a scanning electron microscope (SEM) (S-4800, Hitachi Science Systems, Ltd., Japan) [24].

2.6. Microflora Analysis

2.6.1. DNA Extraction. For the microbial DNA extraction from natural and artificial kefir grains, 10 g of each kefir grain sample was homogenized in 90 ml of sterile peptone solution (0.1% (w/v)) using a Stomacher 400 circulator (Seward Limited, West Sussex, UK) for 15 min at 300 rpm. After the treatment, 1 ml of each grain homogenate was centrifuged at 13000 g for 5 min, the supernatant was removed, and the following procedures were adopted according to the instructions (started with step 3) of PowerFood™ Microbial DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) [25, 26]. Then, PCR amplification was carried out.

The primers 338fgc (5-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG-3′) (the GC clamp is underlined) and 518r (5′-ATT ACC GCG GCT GCT GG-3′) [27] spanning the V3 region of the 16S rDNA gene were used to amplify the bacterial community DNA, while the primer NL1GC (5′-GCG GGC CGC GCG ACC GCC GGG ACG CGC GAG CCG GCG GCG GGC CAT ATC AAT AAG CGG AGG AAA AG-3′) (the GC clamp is underlined) and a reverse primer LS2 (5′-ATT CCC AAA CAA CTC GAC TC–3′) [28] spanning the D1 region of the 26S rRNA gene were used to amplify the yeast community. Besides, the PCR amplification conditions for the bacterial and yeast community were set according to the methods of Han et al. [29] and Cocolin et al. [30], respectively.

2.6.2. DGGE Analysis. The PCR products were analyzed by denaturing gradient gel electrophoresis (DGGE) using a Bio-Rad DCode Universal Mutation Detection System (Bio-Rad, Richmond, CA, USA) according to the methods of (Zhou et al. 2009). These gels were stained for 40 min in a SYBR Green I (Invitrogen, Carlsbad, CA, USA) solution (1 : 10 000, v/v) and then photographed under UV illumination. The dominant DGGE bands were excised and reamplified using the primers without GC clamp [31]. And, the amplification were sequenced using an ABI 3730 XL DNA analyzer (Applied Biosystems, Foster, CA, USA) in Sangon (Shanghai, China). The final sequence results were obtained by analyzing the identity through a GenBank search.

3. Results and Discussion

3.1. Physical Appearance of Kefir Grains and Different Vectors. Physical appearances of natural kefir grains and artificial vectors were observed by photographing (Figure 1). Natural kefir grains were irregularly shaped hard granules, with firm texture and slimy appearance, yellowish-white color, which resemble miniature cauliflower blossoms. They vary in size and are generally between 0.5 and 3.5 centimeters in diameter (Figure 1(a)). It was consistent with the results reported by Ahmed et al. [32]. The encapsulated vector was shaped as translucent jelly with a diameter of 5–8 mm, with no viscosity, odorless, and clear boundaries between grains (Figure 1(b)). The cheese vector was similar to traditional fermented cheese, with certain viscosity and milk flavor (Figure 1(c)). Additionally, the fracture surface of cheese vector was irregularly shaped as cauliflower florets. Thus, in terms of appearance, the cheese vector was more similar to the natural kefir grains than the encapsulated vector.

3.2. Chemical Components of Kefir Grains and Two Vectors. As shown in Table 1, the main components of natural kefir grains were moisture (761.9 ± 10.2 g/kg), polysaccharide (122 ± 17 g/kg), and protein (69.9 ± 4.8 g/kg). Polysaccharide and protein accounted for 51.2% and 29.4% of dry matter, respectively. They were supposed to play an important role in the formation of kefir and in maintaining the structure and special viscoelasticity of kefir granules. As for the two artificial vectors, the moisture of encapsulated vector was much higher, reaching 98%, while the protein content was 1.2% and polysaccharide content was 0%. The components of cheese vector was basically in line with the traditional cheese, containing a moisture content of 59%, a protein content of 26%, and a polysaccharide content of 8%.

Compared with the natural kefir grains, the two kinds of artificial vectors are quite different from natural ones in the content of main components. Nevertheless, the protein and polysaccharide contents of cheese vector were much higher than those of the encapsulated vector. Furthermore, it is speculated that the protein may provide more favorable conditions for the attaching microorganisms and growth of the kefir grains, while the polysaccharides might be the products of microbes in the fermentation process. Thus, the cheese vector seemed more suitable for the growth of grains than the encapsulated vector.

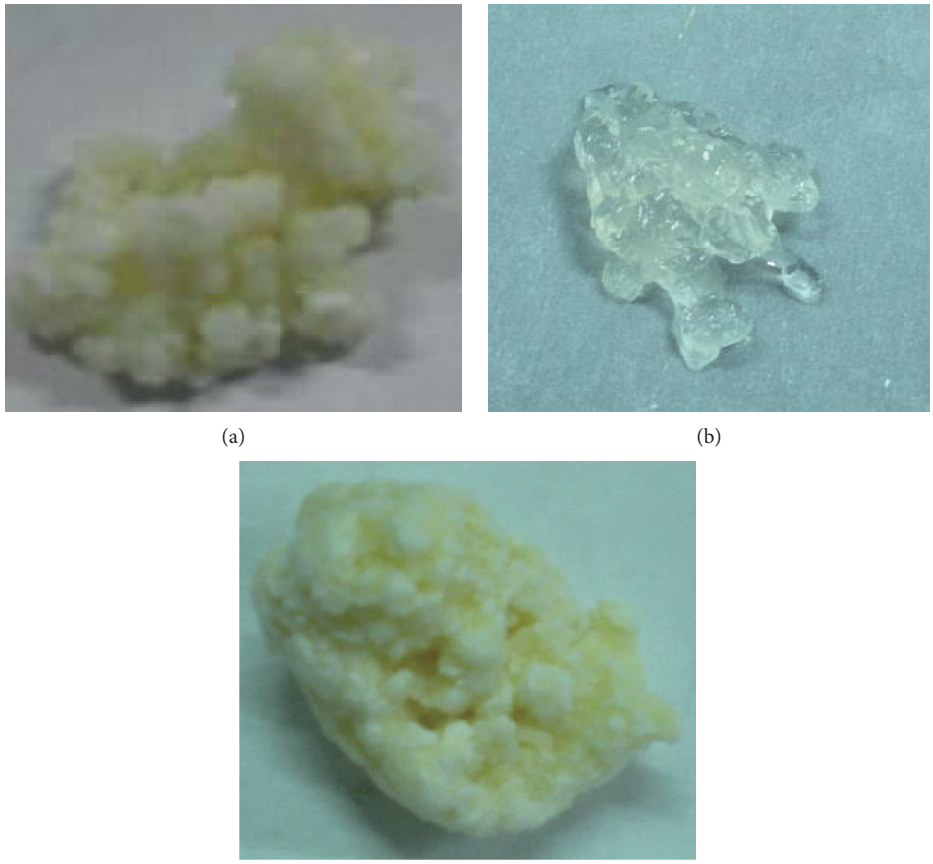
3.3. Microstructure of Kefir Grains and Two Vectors. The external and internal microstructure of kefir grains and artificial vectors were observed by the scanning electron microscope (SEM) method (Figure 2). The flora distribution, quantity, and internal connective matrix of natural kefir grains were different in different regions. Each of them was made up of bacteria and yeasts that adhere to a substrate of kefir grains. The exterior surface of natural kefir grains was smooth and densely populated by lots of short lactobacilli (Figure 2(a)) and nearly no yeasts could be found, while the interior surface was rough, covered with longer lactobacilli and yeasts (Figure 2(b)). There were more bacteria than yeasts, and yeast appeared to be embedded in the bacterial community. Moreover, filamentous appendages were also observed in the interior of kefir grains.

The encapsulated vector was embedded with sporadic yeast and lactobacilli on the exterior surface (Figure 2(c)). The inner layer was more porous and rougher (Figure 2(d)), with filamentous appendages same as that in natural kefir grains. Yet, no bacteria could be observed. It may be due to the fact that the microbes were too dispersed to be found and were affected by the surface tension of encapsulated vector that made the microorganism less and mainly distribute on the outer surface of encapsulated vector. As for microorganisms, the distribution of microorganisms on the outer surface of cheese vector is similar to that of the encapsulated vector, with small number of bacilli distributed on the surface (Figure 2(e)). But the interior surface was rough with some irregular protuberances. The bacteria distribution in the inner cheese vector was similar to the natural grains which were mainly bacillus and a few yeasts. However, there was cheese matrix, but no filamentous appendages existed between bacteria (Figure 2(f)).

The scanning electron microscopy also indicated that the microflora distribution of natural kefir and the two carriers was different. Although lactobacillus and yeast were observed in both carriers, the microflora of the two artificial carriers was less than that of the natural grain. That may result in the shorter time generation of microbes in two artificial vectors. This may be because the two vectors have just been made without passing through generations leading to the lack of mass reproduction of microorganisms.

3.4. Microflora of Kefir Grains and Two Vectors. Kefir grains and two artificial vectors were collected and pretreated. The V3 region of the 16S rRNA gene of bacteria and D1 region of the 26S rRNA gene of yeast were amplified, and the resulting PCR products were analyzed by DGGE (Figure 3).

Figure 3(a) is the fingerprints of the bacterial community in natural kefir grains and two kinds of artificial vectors. Compared with the similar sequences of GenBank, the final sequence results are shown in Table 2. It was clearly



(a) (b) (c)

Figure 1: Physical appearance of natural kefir grains and different vectors: (a) Natural kefir grains; (b) encapsulated vector; (c) cheese vector.

Table 1: Chemical properties of natural kefir grains and different vectors (g/kg).

	Natural kefir grains	Encapsulated vector	Cheese vector
Moisture	$761.23 \pm 8.01^*$	$982.7 \pm 15.2^*$	$589.9 \pm 7.9^*$
Polysaccharide	$122 \pm 17^*$	0^*	$8.2 \pm 2.4^*$
Protein	$69.9 \pm 4.8^*$	$1.2 \pm 0.2^*$	$260.2 \pm 18.8^*$

The data are average values of triplicate ± standard deviation. ∗Mean values on the same line are significantly different ($P < 0.05$).

indicated that the natural kefir grains contained five bands (Figure 3(a)-1) (band A was identified as *Leuconostoc sp.*, B as *Lactobacillus helveticus*, C as *Lactobacillus kefiranofaciens,* D as *Lactococcus lactis*, and E as *Lactobacillus kefiri*) (Table 2), and the cheese vector contained four bands (Figure 3(a)-2), while the encapsulated vector contained five (band F was identified as *Leuconostoc mesenteroides*, G as *Lactobacillus helveticus*, I as *Lactococcus lactis*, and J as *Lactobacillus kefiri*) (Figure 3(a)-3). Due to the low strength of band H, it cannot be removed from the gel and be identified. However, it can be speculated that the H zone is *Lactobacillus Kefiranofaciens* according to the corresponding position in Figure 3(a)-1.

By contrast, there was a slight difference between cheese vector and encapsulated vector. *Leuconostoc mesenteroides* were only present in the encapsulated vector but not in the cheese vector. When compared with the natural kefir grains, the two artificial vectors were found with higher level of *Lactobacillus helveticus* content, while the content of *Lactobacillus kefiranofaciens* was relatively lower.

Figure 3(b) is the fingerprints of the yeast community in natural kefir grains and two kinds of artificial vectors. Figure 3(b)-1 shows that there were mainly 6 different yeasts existing in the natural kefir grains. According to Table 2, the *Kazachstania servazzii* corresponded to the band B, the *Saccharomyces cerevisiae* corresponded to the band C, the *Pichia fermentans* corresponded to the band E, and *Kazachstania unispora* and *Candida inconspicua* corresponded to band A and band D, respectively (Table 2). The reason why bands E and F were corresponding to the same

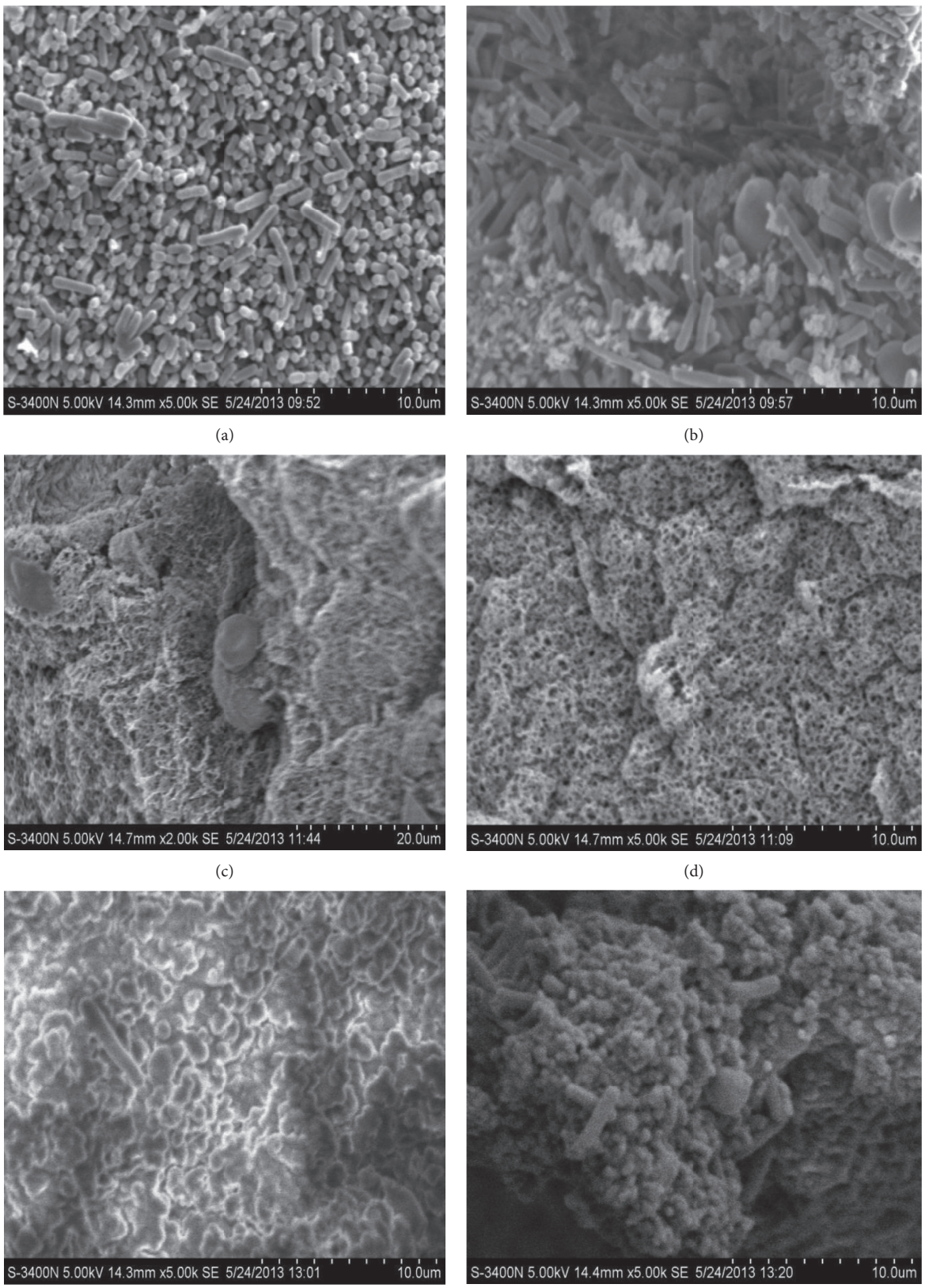


Figure 2: Scanning electron micrographs of natural kefir grains and different vectors: the external structure of (a) natural kefir grains, (c) encapsulated vector, and (e) cheese vector. The internal structure of (b) natural kefir, (d) encapsulated vector, and (f) cheese vector.

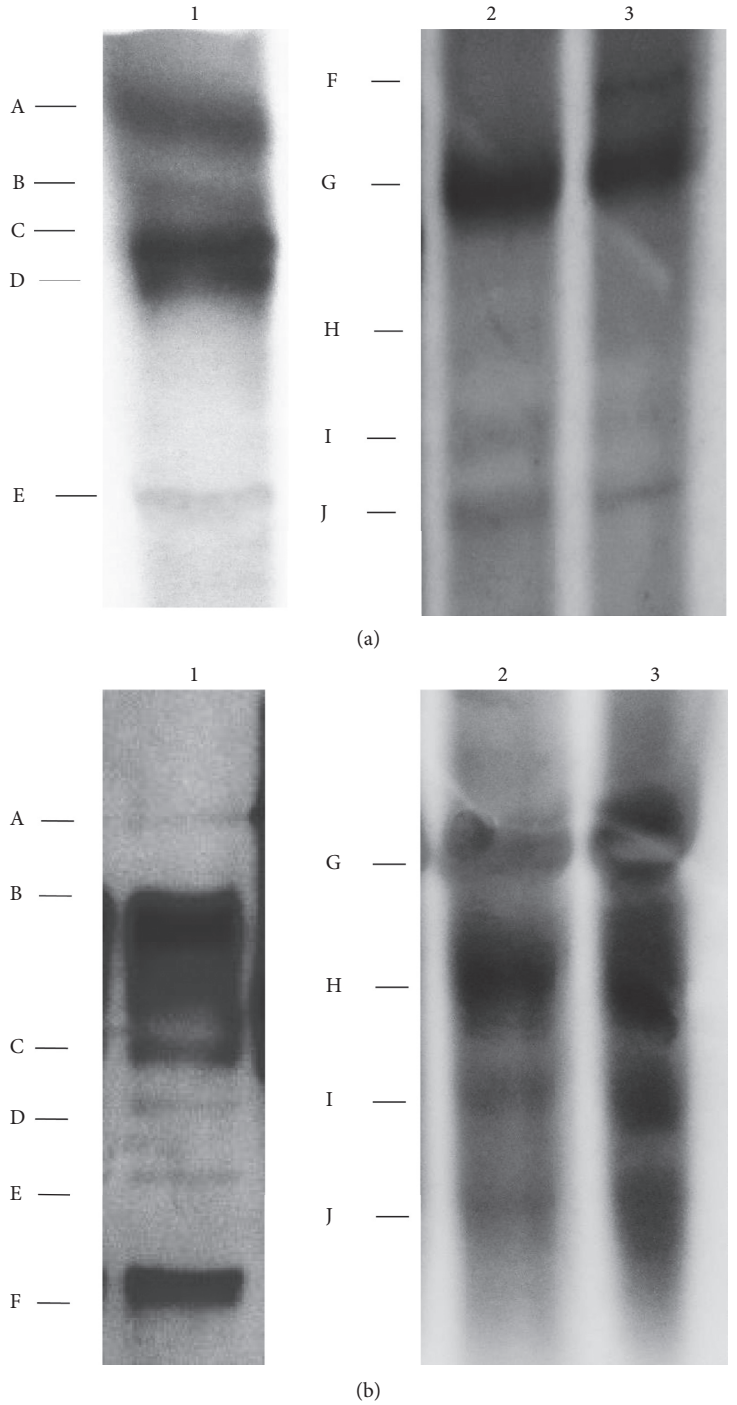


Figure 3: PCR-DGGE fingerprinting of the bacteria and yeast community in natural kefir grains and two different vectors: (a) bacteria community; (b) yeast community. 1 = natural kefir grains; 2 = cheese vector; 3 = encapsulated vector.

strains but different positions might lie in the degradation of the extracted yeast DNA during storage.

Figures 3(b)-2 and 3(b)-3 show that there was no significant difference between the two artificial vectors. Both of them contained four bands, which were identified as *Kazachstania servazzii* (G), *Saccharomyces cerevisiae* (H), *Candida inconspicua* (I), and *Pichia fermentans* (J) (Table 2). When compared with the natural kefir grains, the yeast community in two artificial vectors was almost the same except the lack of *Kazachstania unispora*.

Table 2: PCR-DGGE sequence analysis results of bacteria and yeast community in natural kefir grains and different vectors.

Band*	Strain	Identity (%)**
Bacteria		
A	*Leuconostoc sp.*	98
B	*Lactobacillus helveticus*	99
C	*Lactobacillus kefiranofaciens*	96
D	*Lactococcus lactis*	100
E	*Lactobacillus kefiri*	99
F	*Leuconostoc mesenteroides*	100
G	*Lactobacillus helveticus*	100
I	*Lactococcus lactis*	96
J	*Lactobacillus kefiri*	99
Yeasts		
A	*Kazachstania unispora*	96
B	*Kazachstania servazzii*	99
C	*Saccharomyces cerevisiae*	98
D	*Candida inconspicua*	99
E	*Pichia fermentans*	100
F	*Pichia fermentans*	99
G	*Kazachstania servazzii*	98
H	*Saccharomyces cerevisiae*	100
I	*Candida inconspicua*	99
J	*Pichia fermentans*	99

*Bands are numbered as indicated in the DGGE profiles shown in Figure 3.
**The result of the sequence of the recovered bands was consistent with the recent standard strains in GenBank database (http://www.ncbi.nlm.nih.gov).

4. Conclusions

Various findings indicated that the cheese vector was more similar to the natural kefir grains than the encapsulated vector from the structure, chemical components, microstructure, and microflora aspects. Despite the lack of subculture stability and fermentation flavor experiments, it can be concluded from the above experiments that the cheese vector as a potential kefir grain substitute is better than encapsulated vector to produce kefir milk. However, it still needs to be further researched in the artificial vector growth, the change of microorganism with the growth of vector, and the quality of fermented milk.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declared no potential conflicts of interest with respect to the publication of this article.

Acknowledgments

This work was supported by the National key Research and Development Program (2018YFC1604305-01), National Natural Science Foundation of China (31972972), and the Natural Science Foundation of Heilongjiang Province of China (C2018033).

References

[1] S. Giuseppe, B. Francesca, P. Stefania et al., "Proteomic characterization of kefir milk by two-dimensional electrophoresis followed by mass spectrometry," *Journal of Mass Spectrometry*, 2020.

[2] H. Grønnevik, M. Falstad, and J. A. Narvhus, "Microbiological and chemical properties of Norwegian kefir during storage," *International Dairy Journal*, vol. 21, no. 9, pp. 601–606, 2011.

[3] J. Gao, F. Gu, N. H. Abdella, and H. G. Ruan, "Investigation on culturable microflora in Tibetan kefir grains from different areas of China," *Journal of Food Science*, vol. 77, no. 8, pp. M425–M433, 2012.

[4] M. R. M. Prado, C. Boller, R. G. M. Zibetti, D. de Souza, L. L. Pedroso, and C. R. Soccol, "Anti-inflammatory and angiogenic activity of polysaccharide extract obtained from Tibetan kefir," *Microvascular Research*, vol. 108, pp. 29–33, 2016.

[5] W.-S. Hong, Y.-P. Chen, and M.-J. Chen, "The antiallergic effect of kefir lactobacilli," *Journal of Food Science*, vol. 75, no. 8, pp. H244–H253, 2010.

[6] M. M. Hatmal, A. Nuirat, M. A. Zihlif, and M. O. Taha, "Exploring the influence of culture conditions on kefir's anticancer properties," *Journal of Dairy Science*, vol. 101, no. 5, 2018.

[7] J. He, F. Gu, H. Ruan, Q. Chen, J. He, and G. He, "Induction of apoptosis of gastric cancer cells SGC7901 in vitro by a cell-free fraction of Tibetan kefir," *International Dairy Journal*, vol. 30, no. 1, pp. 14–18, 2013.

[8] S. Plessas, C. Nouska, I. Mantzourani, Y. Kourkoutas, A. Alexopoulos, and E. Bezirtzoglou, "Microbiological exploration of diffferent types of kefir grains," *Fermentation*, vol. 3, no. 1, pp. 1–10, 2017.

[9] J. Piermaría, C. Bengoechea, A. G. Abraham, and A. Guerrero, "Shear and extensional properties of kefiran," *Carbohydrate Polymers*, vol. 152, pp. 97–104, 2016.

[10] B. C. T. Bourrie, B. P. Willing, and P. D. Cotter, "The microbiota and health promoting characteristics of the fermented beverage kefir," *Frontiers in Microbiology*, vol. 7, p. 647, 2016.

[11] W. Liu, M. Zhang, J. Xie et al., "Comparative analyses of microbial community diversities of tibetan kefir grains from three geographic regions," *International Journal of Dairy Technology*, vol. 72, no. 4, pp. 536–544, 2019.

[12] K.-X. Tan, V. N. Chamundeswari, and S. C. J. Loo, "Prospects of kefiran as a food-derived biopolymer for agri-food and biomedical applications," *RSC Advances*, vol. 10, no. 42, pp. 25339–25351, 2020.

[13] N. Habibi, S. Soleimanian-Zad, and M. S. Zeinoddin, "Optimization of kefir grains production by using taguchi technique and mini-fermentation," *World Applied Sciences Journal*, vol. 12, pp. 613–618, 2011.

[14] D. K. Apar, E. Demirhan, and B. Ozel, "Kefir grain biomass production: influence of different cultureing conditions and examination of growth kinetic models," *Journal of Food Process Engineering*, vol. 40, pp. 12332–12341, 2017.

[15] T.-H. Chen, S.-Y. Wang, K.-N. Chen, J.-R. Liu, and M.-J. Chen, "Microbiological and chemical properties of kefir manufactured by entrapped microorganisms isolated from kefir grains," *Journal of Dairy Science*, vol. 92, no. 7, pp. 3002–3013, 2009.

[16] E. R. L. Haydee, P. L. Audry, H. M. Adrián, H. S. Humberto, I. C. S. Patricia, and M. R. A. Rosa, "Probiotic potential of lactobacillus paracasei CT12 isolated from water kefir grains (tibicos)," *Current Microbiology*, vol. 77, pp. 2584–2592, 2020.

[17] L. Wang, H. Zhong, K. Liu, A. Guo, X. Qi, and M. Cai, "The evaluation of kefir pure culture starter: liquid-core capsule entrapping microorganisms isolated from kefir grains," *Food Science and Technology International*, vol. 22, no. 7, pp. 598–608, 2016.

[18] S.-Y. Wang, K.-N. Chen, Y.-M. Lo et al., "Investigation of microorganisms involved in biosynthesis of the kefir grain," *Food Microbiology*, vol. 32, no. 2, pp. 274–285, 2012.

[19] E. Dertli and A. H. Çon, "Microbial diversity of traditional kefir grains and their role on kefir aroma," *LWT-Food Science and Technology*, vol. 85, pp. 151–157, 2017.

[20] E. Sulejmani and A. A. Hayaloglu, "Influence of curd heating on proteolysis and volatiles of Kashkaval cheese," *Food Chemistry*, vol. 211, pp. 160–170, 2016.

[21] C. Soares, A. L. Fernando, N. Alvarenga, and A. P. L. Martins, "Substitution of sodium chloride by potassium chloride in São João cheese of Pico Island," *Dairy Science & Technology*, vol. 96, no. 5, pp. 637–655, 2016.

[22] M. Todaro, M. Palmeri, L. Settanni et al., "Effect of refrigerated storage on microbiological, chemical and sensory characteristics of a ewes' raw milk stretched cheese," *Food Packaging and Shelf Life*, vol. 11, pp. 67–73, 2017.

[23] X.-Q. Zha and J.-P. Luo, "Production stability of active polysaccharides of Dendrobium huoshanense using long-term cultures of protocorm-like bodies," *Planta Medica*, vol. 74, no. 1, pp. 90–93, 2008.

[24] M. Bensmira and B. Jiang, "Effect of some operating variables on the microstructure and physical properties of a novel Kefir formulation," *Journal of Food Engineering*, vol. 108, no. 4, pp. 579–584, 2012.

[25] W. Gao and L. Zhang, "Comparative analysis of the microbial community composition between Tibetan kefir grains and milks," *Food Research International*, vol. 116, pp. 137–144, 2019.

[26] A. M. O. Leite, D. C. A. Leite, E. M. Del Aguila et al., "Microbiological and chemical characteristics of Brazilian kefir during fermentation and storage processes," *Journal of Dairy Science*, vol. 96, no. 7, pp. 4149–4159, 2013.

[27] M. G. d. C. P. Miguel, P. G. Cardoso, L. d. A. Lago, and R. F. Schwan, "Diversity of bacteria present in milk kefir grains using culture-dependent and culture-independent methods," *Food Research International*, vol. 43, no. 5, pp. 1523–1528, 2010.

[28] L. Cocolin, D. Aggio, M. Manzano, C. Cantoni, and G. Comi, "An application of PCR-DGGE analysis to profile the yeast populations in raw milk," *International Dairy Journal*, vol. 12, no. 5, pp. 407–411, 2002.

[29] Y. Han, X. Xu, Y. Jiang, G. Zhou, X. Sun, and B. Xu, "Inactivation of food spoilage bacteria by high pressure processing: evaluation with conventional media and PCR-DGGE analysis," *Food Research International*, vol. 43, no. 6, pp. 1719–1724, 2010.

[30] L. Cocolin, L. F. Bisson, and D. A. Mills, "Direct profiling of the yeast dynamics in wine fermentations," *Fems Microbiology Letters*, vol. 189, no. 1, pp. 81–87, 2000.

[31] W. Gao, L. Zhang, Z. Feng et al., "Microbial diversity and stability during primary cultivation and subcultivation processes of Tibetan kefir," *International Journal of Food Science & Technology*, vol. 50, no. 6, pp. 1468–1476, 2015.

[32] Z. Ahmed, Y. Wang, A. Ahmad et al., "Kefir and health: a contemporary perspective," *Critical Reviews in Food Science and Nutrition*, vol. 53, no. 5, pp. 422–434, 2013.