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PCR-based Identification of Microorganisms in a Kefir Grain

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Abstract Nowadays many people are concerned about being healthy, and many dairy products are taken as health supplementary foods. Among dairy products, kefir, also called as Tibet mushroom, is a yogurt fermented by kefir grain, which is a mixture of lactic acid bacteria and yeasts. Although there are many empirical evidences that kefir is very influential for human body, the exact reason is not definitively discovered. Therefore, it would be useful to understand characteristics of a kefir grain and to categorize bacteria in a kefir grain. In this paper, molecular biological apparatus such as PCR, electrophoresis, PCR purification, DNA sequencing were used to identify and classify the species of lactic acid bacteria and yeast in a kefir grain. We used PCR-based identification method using 16S rRNA primer and Internal Transcribed Spacer (ITS) primer. We identified 6 different species which were selected on different medium. In addition, observation with scanning electron microscope (SEM) enabled us to grasp an external shape of the kefir grain. Although we found a limited number of microbial species, more intensive research are needed for extensive identification of microorganism species in Korean kefir grain.

Key words : Kefir, Kefir grain, 16S rDNA(16S rRNA), PCR-based identification, lactic acid bacteria, Internal Transcribed Spacer (ITS)

Introduction

Kefir is a fermented milk beverage made in parts of Russia, Bulgaria and the former Yugoslavia. It is also called as Tibet mushroom because of its morphological similarity to mushroom. Generally, 24 hours after the inoculation of kefir grain to intact milk, the fermentation process occurs in milk properly by mushroom-like clump of microorganism[14]. Milk is fermented by a mixed and varied population of organisms which usually include *Lactobacilli*, *Lactococcus lactis*, and yeasts, for example, *Saccharomyces* spp. Lactic acid, ethanol and carbon dioxide which causes foaming are the main products. The organisms become embedded in an extracellular polysaccharide (kefiran) to form whitish, gelatinous granules which are carried to the surface by bubbles of carbon dioxide[13]. The granules are collected and used as an inoculum for subsequent fermentations and they can be stored for several days in cold milk or water[15]. Kefir grain is a solid form in which many kinds of *Lactobacilli* are condensed. Many scientists have made clear the exact kinds of some *Lactobacilli*, the others are still on research[6]. Nevertheless, it is obvious that the kefir has many effectiveness like immune enhancement and antibody production[16].

In short, The purpose of this research is extracting different microorganisms from kefir using molecular biological experiments, and analyzing population of microorganisms in kefir to verify the beneficial effects of kefir by comparing the population of it to that of other lactic acids fermentation.

Two ways are commonly used to classify microorganisms. The first is a traditional way using morphological, chemical, and physiological traits. In Bergey's Manual of Systematic Bacteriology, the main factors for classification are gram staining, cell morphology and arrangement, and aerobic fermentation. Another uses nucleic acids and proteins[8]. The other one is a molecular biological way using DNA sequence.

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DNA hybridization, comparing contents of G and C bases, and analysis of DNA sequence are mainly used methods for identification. Among these, analysis of 16S and 18S rDNA sequence is main avenue for this research. For yeasts, sequencing of ITS (Internal Transcribed Spacer) region is used. The reason using rDNA region is rRNA is transcribed from rDNA. Moreover, rRNA is an essential element of ribosome which produces the protein from mRNA.

rDNA of small subunit rRNA(SSU rRNA), especially 16S rDNA, is mostly used for identification of prokaryotes. Because amplification of rDNA and sequencing is available, it is easy to identify for unknown microorganism[7]. For eukaryotes, they have 3 closed rDNA regions, 18S, 5.8S, 28S, and among these regions ITS 1 and ITS 2 exist[3]. They are used importantly in identification of eukaryotes like 16S rDNA region of prokaryotes. In short, to get specific sequences of bacteria, amplifying 16s rDNA part makes more convenient not only to amplify microflora of a kefir grain but also to shorten the time for the process[10]. Almost all of bacteria DNAs have very similar but not exactly same 16s rDNA region. That's the key to identify each species of bacteria. In this research, we extracted different types of microorganism contained in kefir and classified them with molecular biological tools using rRNA sequences and ITS between rRNAs.

Materials and Methods

Cultivation

In this research, KSAK1 was obtained from internet kefir club (http://cafe.naver.com/.kefir) and was cultivated and maintained by subculture in milk. We used four main sorts of solid culture medium. They were MRS-agar[4], Yeast extract-glucose chloramphenicol-agar (YGC-agar)[6], Lee medium[11], and acetic acid bacteria medium (AAB medium)[2]. MRS-agar was purchased from Difco, USA. YGC-agar, Lee medium, and AAB medium were made following previous articles. We mixed tryptone 10, yeast extract 10, lactose 5, sucrose 5, $CaCO_3$ 3, K_2HPO_4 0.5, bromocresol purple 0.02, agar 18 (g/ℓ) for Lee medium, yeast extract 30, BCG 0.2, ethanol 20, agar 20 (g/ℓ) for AAB (Acetic acid bacteria) medium, and bacto yeast extract 5, glucose 20, chloramphenicol 0.1, bacto agar 13 (g/ℓ) for YGC-agar.

Different medium was used for the selective cultivation of each species of bacteria. MRS-agar was used for *lactobacillus*, YGC-agar for *saccharomyces*, Lee medium *lactococcus*, AAB medium acetic acid bacteria. For streaking to plates, inoculating loop was put into a grain of KSAK1.

PCR amplification

After the cultivation process, colonies were selected regarding various sizes of colonies in plates. For instance, in the Lee medium plate, 3 different colors of colonies are cultivated. 10 colonies from 4 plates were totally selected to be used for experiments. The colonies were named as MRS Big, MRS Small, AAB Small, Lee Biggest, Lee Medium, Lee Smallest, Lee Yellow, Lee Middle Color, YGC Big, and YGC Small regarding their plates and sizes and colors. Colonies were used directly in PCR reaction. Total volume of PCR reaction was 20 $\mu\ell$ and included a small amount of a colony, 2$\mu\ell$ of 10x Buffer, 0.6$\mu\ell$ of dNTP mix, 3.2$\mu\ell$ of primer, 0.2$\mu\ell$ of Taq Polymerase, 14$\mu\ell$ of DDW. PCR reaction with a bacterial colony was performed as follows; The first denaturation cycle at 95℃ for 4 minutes, 40 cycles of 95℃ for 20 seconds, 55℃ for 20 seconds, and 72℃ for 30 seconds, and final elongation cycle at 72℃ for 5 minutes. PCR reaction with an yeast colony was same as above except annealing at 45℃.

In this research, 519f and 1492r for bacteria and ITS3 and ITS4 for yeasts were used as previous report[3]. Primer sequences are provided in Table 1. Because it is unknown that what colonies include bacteria or yeasts, the first PCR process is done for all the 10 colonies with both primers. It is strongly predicted YGC Big and YGC Small have yeasts because YGC-agar (Yeast extract-glucose chloramphenicol-agar) is specialized for yeast cultivation. Amplified sequences are observed by electrophoresis using 1.1% agarose gel. From the results of electrophoresis experiments, we selected colonies which would be analyzed by DNA sequencing. Besides with this results, we repeated electrophoresis processes selectively for amplification of all 10 colonies.

DNA Purification and sequencing

After checking the amplification results, we purified amplified PCR products. The purification process was performed with PCR Purification Kit (Solgent, Korea).

Table 1. Primers sequences used in this research

	Primer name	Sequences	mer
prokaryote	Forward 519f[1)]	5'-CAGCMGCCGCGGTAATWC-3'	18
	Reverse 1492r[2)]	5'-GGTTACCTTGTTACGACTT-3'	19
eukaryote	ITS3[3)]	5'-GCATCGATGAAGAACGCAGC-3'	20
	ITS4[3)]	5'-TCCTCCGCTTATTGATATGC-3'	20

1) 519f: 519-536 of *E. coli* 16S rRNA numbering[1, 9, 10]
2) 1492r: 1510-1492 of *E. coli* 16S rRNA numbering[5, 10]
3) ITS3, ITS4: partial sequence of *S. cerevisiae* Internal Transcribed Spacer 2[3, 12]

DNA sequencing process was requested to an expertise sequencing company, Solgent (Daejeon Korea). Homology searches were conducted with sequences in the GenBank/EMBL/DDBJ database with the BLAST program.

Morphological Observation

Although this research did not identify bacteria in the KSAK1 by the traditional way, observing the KSAK1 kefir using ESEM (Quanta 200, Netherlands) was performed to grasp an external shape of KSAK1. In this research, SEM pictures were taken with (LFD) large field detector, which is not ESEM mode but also takes pictures of wet materials easily.

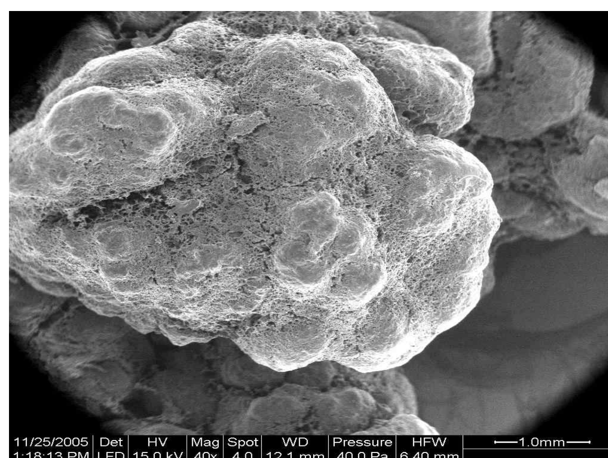


Fig. 1. Morphological Shape of Kefir (40x).

Results

External Shape of KSAK1

Kefir grain is encapsulated by a protein layer called kefiran. Some bacteria protrude the layer, so we could see some shape of bacteria outside kefiran. The pictures taken by ESEM are shown from Fig. 1 and Fig. 2 It was not easy to take very accurate picture because it was quite difficult to cut the kefir grain properly. We could note that the surface of the grains is different from the inner surface of the grains.

Two aggregations of bacteria located in right center in Fig. 2. Kefiran dominantly covered almost all of surface of a kefir grain. However, kefiran was not smooth and flat. Some parts of kefiran were very sparsely connected with long and thin protein or polysaccharide masses. In summary, kefiran was a main capsulated structure that composes basic architecture, and some bacteria appeared outside kefiran.

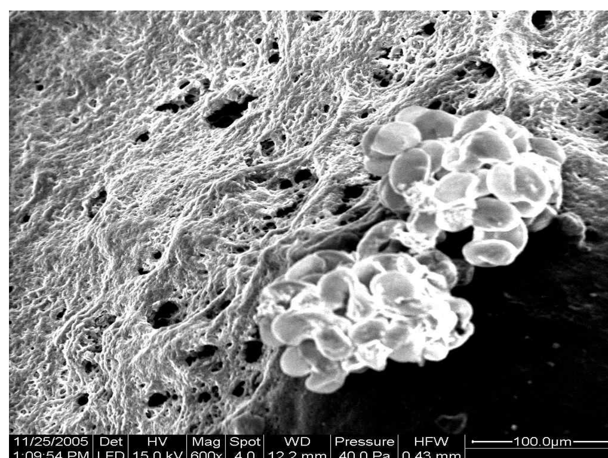
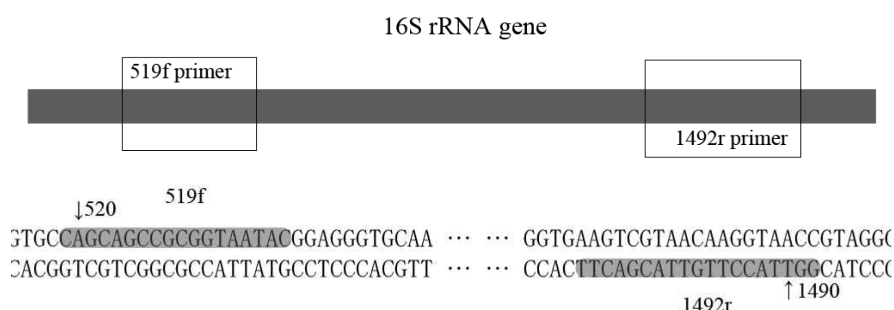


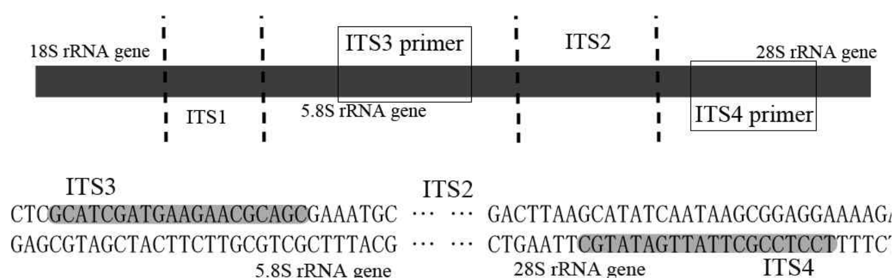
Fig. 2. Bacteria Shape outside Kefiran (600x).

PCR

Two different sets of primers were used in this report for amplification of bacterial 16S RNA and Eucaryotic ITS region as shown in Fig. 3. We labeled a number for each colony like MRS Big as 1, MRS Small as 2, AAB Small as 3, Lee Biggest as 4, Lee Medium as 5, Lee Smallest as 6, Lee Yellow as 7, Lee Middle Color as 8, YGC Big as 9, and YGC Small as 10. In addition to primers targeting bacteria abbreviated to B, primers targeting yeasts were abbreviated to Y. So, for example, Lee Medium amplified with 519f and 1492r



A. The location of primer 519f and 1492r in *E. coli* genome



B. The location of primer ITS3 and ITS4 in *S. cerevisiae* genome

Fig. 3. Schematic location of primers used in this research

abbreviated to B5. The electrophoresis was performed to check the PCR results (Fig. 4). The first ten lanes except size marker were from B1 to B10 and the last ten lanes were from Y1 to Y10. As a result, Y1, Y2, Y3, Y4, Y5, Y8, and Y9 (300-500 bp) were amplified by yeast primers. However, Y10 was not amplified by yeast primers and colones from 1, 2, 3, 4, 5 and 8 were amplified with yeast primers although only colony from 9 was only from YGC-agar.

DNA Purification

As a summation, Y1, Y2, Y3, Y4, Y5, Y8, Y9, B3, B4, B5, B6, Y7, and B10 were amplified and purified to be analyzed for their DNA sequences (Fig. 5). B4 and B10 bands were nearly invisible. At last, B4 and B10 could not be analyzed by sequence analysis. The amplified PCR products showed similar length of DNA around 900 bp for bacterial primer set and around 400 bp for yeast primer set.

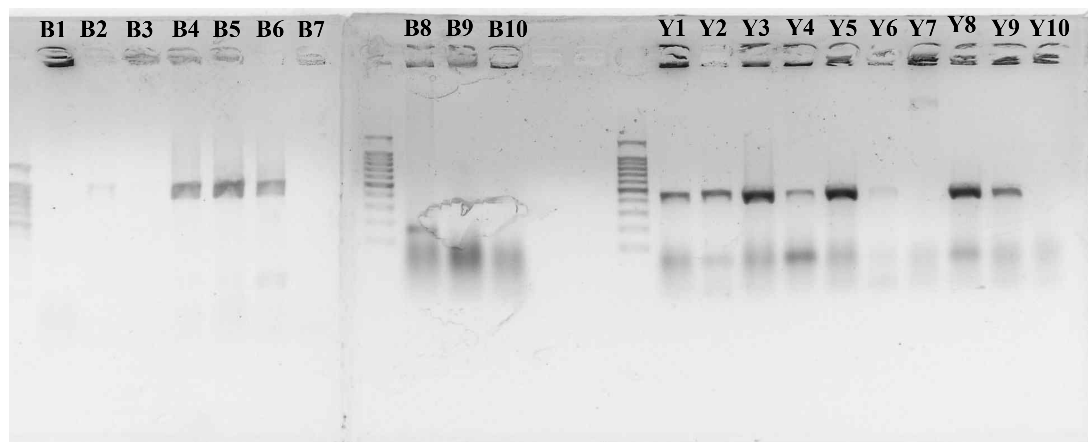


Fig. 4. The PCR result

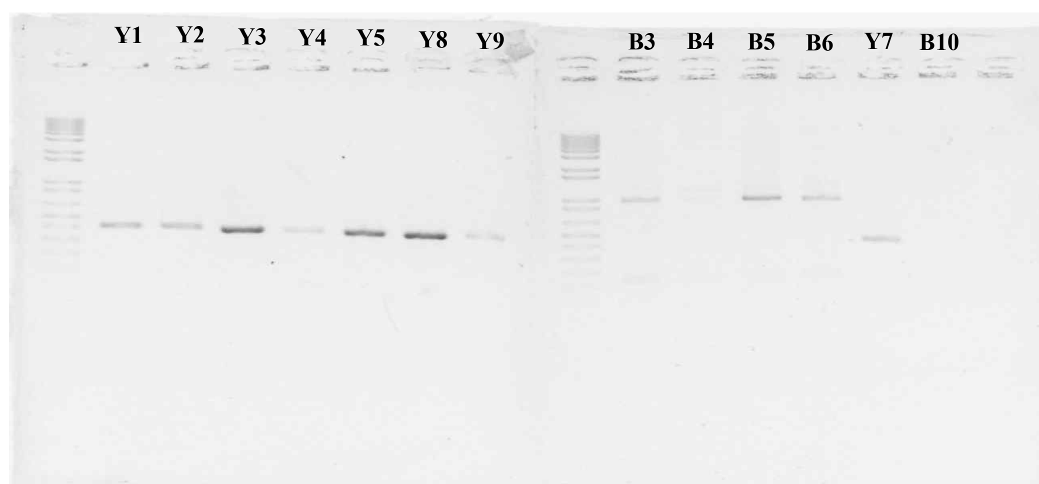


Fig. 5. Electrophoresis after PCR Purification

Sequencing analysis can extend to about 500-600 bp per a sequencing reaction. Because amplified 16S rDNA of bacteria was generally 900 bp, two primers were used to read the sequence from two opposite ends. For example, in this research two primers, 519f and 1492r, were used for bacteria sequencing. 519f was used to read sequences in forward direction, 1492r was used in reverse direction[10]. On the other hand, one primer was enough for sequencing of yeasts because amplified ITS region of yeast was only about 500 bp.

Identification

The NCBI (National Center for Biotechnology Information) site, *http://www.ncbi.nih.gov*, was used for identification of bacteria and yeasts. Because two primers were used for bacteria, we compared the result of 519f based sequencing and the result of 1492r based sequencing. The best species that showed the highest matching rate was selected.

As a result of identification, at least 4 species of

Table 2. Result of identification of KSAK1

Object	Sample (Medium)	Major populations detected	Similarity(%)to known sequences
KSAK1	B3 (AAB)	*Lactobacillus sp.* CIDCA 8348 *Lactobacillus kefiri*	100 99
	B5 (Lee)	*Lactobacillus sp.* CIDCA 8348 *Lactobacillus kefiri* *Lactobacillus parabuchneri*	99 99 99
	B6 (Lee)	*Lactobacillus sp.* CIDCA 8348 *Lactobacillus kefiri*	99 99
	Y1 (MRS)	*Saccharomyces unisporus*	99
	Y2 (MRS)	*Saccharomyces humaticus* *Saccharomyces turicensis*	99 99
	Y3 (AAB)	*Saccharomyces unisporus*	99
	Y4 (Lee)	*Saccharomyces unisporus*	94
	Y5 (Lee)	*Saccharomyces unisporus*	99
	Y7 (Lee)	*Saccharomyces humaticus* *Saccharomyces turicensis*	100 100
	Y8 (Lee)	*Saccharomyces unisporus*	99
	Y9 (YGC)	*Saccharomyces unisporus*	99

microorganisms appeared in KSAK1. From B3, B4, B5, B6, main species of bacteria were *Lactobacillus sp.* and *Lactobacillus kefiri*. *Saccharomyces unisporous* and *Saccharomyces humaticus* were main species of yeast in KSAK1. For elaborated explanation, Y1, Y3, Y4, Y5, Y8, Y9 showed 100% matching sequence to *Saccharomyces unisporous*, and Y2 and Y7 showed 99% matching sequence to *Saccharomyces humaticus*. The result was shown in Table 2. Because some different kinds of bacteria showed same matching rates for their sequences, it was impossible to decide only one species and all the candidates were provided in the Table 2. The concentration of amplified B4 and B10 was too low to be analyzed by sequencing analysis.

Discussion

At least 6 species of microorganisms were identified through this experiment. The bizarre fact was that the identification results did not rely on medium. As it is mentioned in Materials and Methods, each medium was specified to cultivate different genus of bacteria, but all genus of bacteria identified in this research were *Lactobacillus.* For instance, B3 was from AAB-agar and found to be *Lactobacillus* albeit AAB-agar was optimized for cultivation of acetic acid bacteria. Although the colonies from AAB plate were very small and rare, bacteria other than acetic acid bacteria could grow on this medium. It also applied to other medium.

For yeasts, *Saccharomyces unisporous* appeared in all of the colonies except Y2 and Y7. It was considerable that *Saccharomyces unisporous* was very strongly productive in KSAK1, which was a typical type of Korean kefir grains. If *Saccharomyces unisporous* had its uniquely beneficial trait, its application into Korean health foods such as toenjang, and fermented soy beans would be conducive.

For both bacteria and yeasts, the size of a colony means nothing about the species that comprises the colony. The result showed that, regardless of size of colony, *Saccharomyces unisporous* was identified in case of yeasts. On the other hand, we should consider about the relationship between color of a colony and its species. Y7 (yellow colony in Lee medium) showed different species, *Saccharomyces humaticus* or *Saccharomyces turicensis,* compared to other colonies in Lee culture medium.

In conclusion, main microflora of KSAK1, a typical Korean kefir grain, was discovered to be *Lactobacillus* and *Sacchromyces* and it was not quite different from general kefir grains in other sites.

References

1. Brosius, J., Palmer, M. L., Kennedy, P. J., and Noller, H. F. 1978. Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. *Proc Natl Acad Sci USA*, **75**, 4801-4805.
2. Carr, J. G. and Passmore, S. M. 1979. Methods for identifying acetic acid bacteria. In: Identification methods for microbiologist (Eds. Shinner, F. A. and Lovelock, D. W.). London: Academic Press, Inc.
3. Chen, Y. C., Eisner, J. D., Kattar, M. M., Rassoulian-barrett, S. L., Lafe, A., Yarfitz, S. L., Limaye, A. P., and Cookson, B. T. 2000. Identification of medically important yeasts using PCR-based detection of DNA sequence polymorphisms in the internal transcribed spacer 2 region of the rRNA genes. *J. Clin. Microbiol.* **38**(6), 2302-2310.
4. De Man, J. C., Rogosa, M., and Sharpe, M. 1960. A medium for cultivation of *lactobacilli*. *J. .Appl. Bacteriol.* **23**, 130-135.
5. Eden, P. A., Schmidt, T. M., Blakemore, R. P., and Pace, N. R. 1991. Phylogenetic analysis of *Aquaspirillum magnetotacticum* using polymerase chain reaction-amplified 16S rRNA-specific DNA. *Int. J. Syst. Bacteriol,* **41**, 324-325.
6. Garrote, Graciela L., Abraham, Analia G., and De Antoni, Graciela L. 2001. Chemical and microbiological characterization of kefir grains. *J. Dairy Res.* **68**, 639-652.
7. Hiraishi, A. 2004. *Biseibutsu no Bunrui Doutei Jikkenhou.* (Eds. Suzuki, K., Hiraishi, A., and Yokota, A.). Tokyo: Springer-Verlag Tokyo, Inc.
8. Krieg N. R. and Holt J. G. 1984. Bergey's Manual of Systematic Bacteriology, Vol. 2. Williams & Wilkins Baltimore/London pp 1235-1245.
9. Lane, D. J. Pace, B., Olsen, G. J., Stahl, D. A., Sogin, M. L., and Pace, N. R. 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proc Natl Acad Sci USA* **82**, 6955-6959.
10. Lee, Ji-Young, Jeong, Eun-Young, Lee, Kyu-Sang, Ju, Seul, Kim, Jong-Bae, Kang, Joon-Wun, and Lee, Hyeyoung. 2003. Development of a Monitoring System for Water-borne Bacteria by a Molecular Technique, PCR-RFLP-sequence Analysis. *J. Biomed. Lab. Sci.* **9**, 139-144.
11. Lee, S., Vedamuthu, E., Washam, C., and Reinhold, G. 1974. An agar medium for the differential enumeration of yogurt starter bacteria. *J. Milk Food Technol.* **37**, 272-276.
12. Lott, T. J., Burns, B. M., Zancope-Oliveira, R., Elie, C. M., and Reiss, E. 1993. Nucleotide sequence analysis ofthe 5.8S rDNA and adjacent ITS 2 region of *Candida*

albicans and related species. *Yeast* **9**, 1199-1206.
13. Marshall, V. M. 1993. Starter cultures for milk fermentation and their characteristics. *J. Soc. Dairy Technol.* **46**, 49-56.
14. Saloff-Coste, C. J. 1996. Kefir. Nutritional and health benefits of yogurt and fermented milks. *Danone World Newsletter* **11**, 1-7.
15. Singleton, P. and Diana Sainsbury, Dictionary of Microbiology and Molecular Biology, 3rd Edition, John Wiley and Sons, Ltd., 2001.
16. Vinerola, C. G., Duarte, J., Thangavel, D., Perdigon, G., Farnworth, E., and Matar, C. 2004. Immunomodulating capacity of kefir. *J. Dairy Res.* **72**, 195-200.