Identification of bacteria and yeast communities in a Thai sugary kefir by Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) analyses

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Abstract

The microbial diversity of Thai sugary kefir was identified for the first time by Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) analysis. The V3 regions of 16S bacterial rDNA and 18S rDNA of yeasts in both kefir grains and kefir fermentates were analyzed. Acetic acid bacteria *Gluconobacter japonicus* and *Bacillus cereus* were identified with 99% similarity to the GenBank database. Other bacteria species, including *Lactobacillus rhamnosus*, were also identified with less than 97% similarity. Two yeast species, namely *Saccharomyces cerevisiae*, *Candida ethanolica*, were identified with 99% similarity to the GenBank database. Sugary kefir grains and fermentates have higher diversity of bacteria communities than yeast communities. In comparison with the microorganisms found in sugary kefir cultured in different parts of the world, *Lactobacillus paracasei, Lactobacillus rhamnosus, Bacillus cereus*, and *Saccharomyces cerevisiae* were the common species also found in a Thai kefir. This study is the first time to find the yeast *Candida ethanolica* in sugary kefir. Morphology of sugary kefir grains revealed that bacteria and yeasts spread throughout the grains. Yeast communities are located on the outer portion of kefir grains and the bacterial communities are located on the inner portion of kefir grains.

Keywords : Sugary kefir, PCR-DGGE analysis, Microbial community, *Gluconobacter japonicus*; *Saccharomyces cerevisiae Candida ethanolica*

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1. Introduction

Kefir is an acid-alcoholic fermented beverage which was originated in the Caucasus Mountains [1-3]. Typically, there are two kinds of kefir including milk kefir and sugary kefir. Milk kefir is made of culturing kefir grain in dairy products. In addition, kefir grain can be cultured in sweetened solution or sugar solution which is known as sugary kefir [4], water kefir [5], or tipico [6]. Sugary kefir and milk kefir differ in their substrate, and kefir grain characteristics [7]. Milk kefir grain is small white cauliflower shape while a grain of sugary kefir is brown and transparent.

Kefir grain consists of bacteria and yeasts that are held together in symbiosis with polysaccharides and proteins [2-3]. The major groups of bacteria in kefir are lactic acid bacteria *Lactococcus*, *Lactobacillus*, and *Streptococcus*, followed by acetic acid bacteria [5, 8-9]. Yeasts found in kefir are mostly from the genus *Saccharomyces*, *Candida*, *Kluyeromyces*, and *Pichia* [2, 4, 6, 10-12]. Bacteria and yeasts in kefir have the ability to produce biomass and metabolite products which include organic acids (lactic acid, acetic acid, propionic acid, and butyric acid), CO_2, and ethanol. Since microbial composition found in kefir is considered as a probiotic, consumption of kefir products could give human health benefits [13].

Several studies of microbial composition in milk kefir and sugary kefir from many regions such as Spain [2], Germany [5], Belgium [14], Ireland [15], Turkey [16], Brazil [17], Argentina [8], and Taiwan [18] have been reported. Different microbial species were found in kefir beverages cultured in different parts of the world. In Thailand, kefir is cultured and consumed in household in order to provide health benefit. There was a study that revealed microbial genus composition of milk kefir [19]. However, the information on the microbial community in sugary kefir grains and fermentate found in Thailand has not yet been identified. Therefore, microbial species of sugary kefir found in Thailand should be identified in order to provide guidelines during sugary kefir drinks production and facilitate the verification of potential health benefits from the consumption of Thai sugary kefir drinks.

In this study, a sugary kefir from Phuket, a southern province of Thailand was cultured in a sucrose media and the PCR-DGGE technique was used to determine the bacteria and yeast communities present in sugary kefir grains and fermentate. Microbial distribution in kefir grain was observed by scanning electron microscope (SEM).

2. Materials and methods

2.1 Kefir inocula

A sugary kefir grains were collected from a household in Phuket, a southern province of Thailand. The kefir grains have been cultured in 5% (w/v) sucrose solution (brown sugar) in mineral drinking water. The kefir grains were separated through a cloth-filter and transferred to a fresh media every 3 days.

Kefir grain inocula were kept at 4 °C before the analysis.

2.2 Culture medium and fermentation conditions

Sucrose (from brown sugar) was used as the only carbon source. It was prepared by dissolving 3% (w/v) of brown sugar in water (100 mL) in 125 mL Erlenmeyer flasks and di-ammonium hydrogen orthophosphate (DAP, 2.6% w/v) was used as the nitrogen and phosphorus sources [20]. The medium was autoclaved at 121 °C and 15 psi for 15 min prior to usage. Sugary kefir grains of 0.10 g in wet weight (corresponding to ~ 0.033 g in dried weight) were cultured in the sucrose culture medium. The experiments were carried out under repeated-batch fermentation at room temperature (25 °C – 28 °C) for 30 days. Media (100 mL) were replaced every 3 days. The kefir grains and fermentate were collected through a vacuum-filter and kept at 4 °C prior to the analysis.

2.3 DNA extraction

The kefir grains and fermentate from sucrose fermentation were subjected to DNA extraction by CTAB method [21]. Kefir grains (1g in wet weight) were briefly frozen in liquid nitrogen and ground using a sterile mortar. Aqueous fermentate (10 mL) were harvested by centrifuging at 10,000 x g for 10 min. The precipitates were frozen with liquid nitrogen and ground with mortar. CTAB buffer (2% hexadecyltrimethylammonium bromide (CTAB), 100 mM Tris-HCl [pH 8], 20 mM EDTA, 1.4 M NaCl, 800 µL) was added immediately and incubated for 1 hr at 60 °C. After incubation for 10 min at ambient temperature, 800 µL of a chloroform/isoamyl alcohol solution (24/1, v/v) was added. The tubes were gently stirred and centrifuged at 4 °C for 10 min at 14,000 **X** g. The supernatant was harvested into a new sterile micro-centrifuge tube. 2-propanol (600 µL) was added to the supernatant. The supernatant was left to precipitate at ambient temperature for 2 hr. The tubes were centrifuged at 4 °C for 15 min at 14,000 **X** g to form pellets, and the supernatant was eliminated. Pellets were resuspended and washed twice with 1 mL of ice-cold 70% ethanol in water and centrifuged at 4 °C for 15 min at 14,000 **X** g. The DNA pellets were dehydrated by leaving the caps of the tubes open at ambient temperature for 10 min. Sterilized DI water (30-50 µL) was then added to suspend the pellets. The suspended pellets were preserved at -20 ºC for subsequent analyses. The qualities of genomic DNA were determined by running 2 µL of DNA template on 1% (w/v) agarose gel under the following conditions: 1 x TAE buffer at 100 V for 30 min, and observed under UV light. Clear bands of genomic DNA are observed at the high molecular weight zone of DNA marker.

2.4 PCR amplification

Genomic DNA was further amplified by Polymerase Chain Reaction (PCR) technique. A reaction volume (50 µL) of PCR contained 25 µL of

Taq-DNA polymerase mixture, 1 µL of 10 µM of each primer, 2 µL of DNA template (diluted to 50 ng/µL), and sterilized deionized water (21 µL). PCR amplification was done in a Thermal Cycler (Bio-Rad C1000TM, Richmond, CA, USA). The PCR condition was modified from the previously published method by Magalhaes et al. (2010) [17]. The optimum PCR conditions of bacteria and yeast communities for sugary kefir grains and fermentate samples are shown in Table 1.

Primers used in this study are shown in Table 2. The primers are normally used as a starting point of a target DNA synthesis. In order to identify the bacteria, extracted genomic DNA was applied as the template for amplifying the V3 region of 16S rDNA. The forward primer 338fGC and the reverse primer 518r were used [17]. For the yeast isolates, the forward primer NS3GC and the reverse primer YM951r were used to amplify 18S rDNA gene [22].

Table 1 PCR conditions of bacteria and yeast communities for sugary kefir grain and fermentate samples

Microbial community	Sample	Number of cycles	PCR conditions
Bacteria	Grain	30	Initial denaturation: 95˚C, 5 min,
	Fermentate	30	Denaturation: 92˚C 1 min,
			Annealing: 55˚C 1 min,
			Extension: 72˚C, 1 min,
			Final extension: 72˚C, 10 min.
Yeast	Grain	30	Initial denaturation; 95˚C, 5 min,
	Fermentate	35	Denaturation: 92˚C, 1 min,
			Annealing: 49˚C, 1 min,
			Extension: 68˚C, 1 min,
			Final extension: 68˚C, 10 min.

2.5 DGGE analysis

The Denaturing Gradient Gel Electrophoresis (DGGE) method was performed to isolate PCR fragments. The procedures were adopted from Magalhaes et al. (2010) [17]. PCR fragments (50 µL) were separated on 8% (w/v) polyacrylamide gels in 1 x TAE buffer. The gradient of denaturant (urea and formamide) for bacterial and yeast were 30-55% and 40-60%, respectively. The 100% of denaturant is equal to 7 M of urea and 40% (v/v) of formamide.

Electrophoresis was operated at 85 V, 60 ºC for 10 h and 12 h, for bacteria and yeast, respectively. The gels were stained with ethidium bromide for 15 min and visualized by UV transillumination (UV Transilluminator 2000, Bio-RAD, CA, USA). The images were captured using a Gel Doc Biorad Molecular Imager ChemiDoc XRS+ Systems (Bio-Rad, USA). The selected DGGE fragments were cut from the gels and were separated out with the NucleoSpin Gel and PCR Clean-up (Macherey-Nagel, Düren, Germany). In order to obtain a sufficient amount of DNA for sequencing analysis, the eluted DNA of each band was re-amplified using the same primer without GC clamp [17, 22] (Table 2).

Table 2 Primers for PCR and PCR-DGGE protocols for bacteria and yeast communities

Microbial community	**Universal Primer**		**References**
	PCR	**PCR-DGGE**	
Bacteria	*Forward primer: 338fGC* (5'-CGCCCGCCGCGCGCG GCGGGCGGGGCGGGGGCACGGG GGGACTCCTACGGGAGGCAGCA G-3')	*Forward primer: 338f* (5'-ACTCCTACGGGAAGCAG-3')	[17]
	Reverse primer: 518r (5'-ATTACCGCGGCTGCT GG-3')	*Reverse primer: 518r* (5'-ATTACCGCGGCTGCTGG-3')	
Yeast	*Forward primer: NS3GC* (5'-GCCCGCCGCGCGCGGCGGGCGG GGCGGGGCAAGTCTGGTGCCAG CAGCC-3')	*Forward primer: NS3* (5'-GCAAGTCTGGTGCCAGCAGCC-3')	[22]
	Reverse primer: YM951r (5'-TTGGCAAATGCTTTCGC-3')	*Reverse primer: YM951r* (5'-TTGGCAAATGCTTTCGC-3')	

2.6 DGGE sequencing analysis

The DGGE fingerprints were re-amplified by PCR with the primers listed in Table 2. The PCR amplicons were sent for sequencing at Macrogen Inc. (Seoul, South Korea). DNA sequences were identified based on the nucleotide BLAST database of GenBank (http://www.ncbi.nlm.nih.gov/BLAST/) to identify the closest species from reference DNA sequences.

2.7 Microbial morphology in kefir grain by SEM analysis

Morphology of sugary kefir grains cultured in sucrose solution was observed with a scanning electron microscope (SEM, JSM-5410LV, JEOL Ltd., Japan), according to the modified method [17]. In brief, the grains were submerged in 0.1 M phosphate buffer solution (PBS) containing 2.5% glutaraldehyde at pH 7.2 for 1-2 hr. The samples were rinsed twice with PBS buffer and once with distilled water for 10 min. The sample was then dehydrated in ethanol series of 30, 50, 70, 95, and 100% (v/v) for three cycles. Samples were dried with a critical point dryer (Quorum K850, UK) and coated with gold (sputter coater, Balzers SCD 040, Balzers Union, Liechtenstein).

3. Results and discussion

3.1 DNA extraction

Genomic DNA was extracted from kefir grains and fermentate cultured in the sucrose media. The extracted genomic DNAs for both kefir grains and fermentate samples with high molecular weight were observed above 3,000 bp (base pairs) of DNA marker. The other parts of the extracted DNA contain unstable low molecular weight genetic material RNA and proteins, which did not affect the quality of DNA. An unstable RNA with short half-life in extracted genomic DNA mixture was also found by Tan and Yiap (2009) [23]. The genomic DNA was successfully extracted for further PCR amplifications.

3.2 PCR amplification

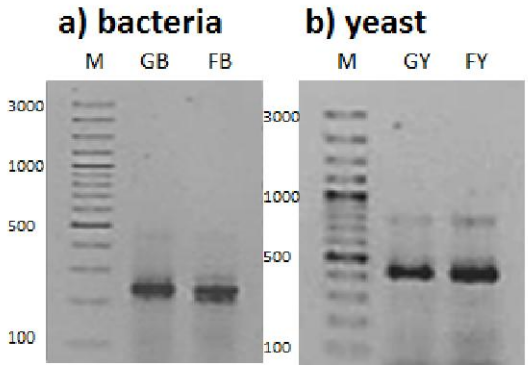

Fig. 1. Profile of PCR products for a) bacteria and b) yeast; M = DNA marker 100-3,000 bp, GB= bacteria in grain, FB= bacteria in fermentate, GY= yeast in grain, FY= yeast in fermentate.

Genomic DNA samples of kefir grains and fermentate were amplified with universal primers for bacteria and yeast using PCR technique [12, 24]. The genomic DNA was amplified for the 16S rDNA (V3 region) for the bacterial community analysis and the

target for the yeast community analysis was the 18S rDNA region. The PCR product of bacteria and yeast are shown in Fig. 1. The bacterial PCR products of kefir grain (GB) and fermentate (FB) are shown as a single band in the same length of 200-300 bp which is the targeted size for the V3 region of 16S rDNA, while the PCR products for yeast are larger in size. The targeted size of PCR product for 18S rDNA is around 400-500 bp. Yeast PCR products for both kefir grain (GY) and fermentate (FY) are 400-500 bp (Fig. 1b). The PCR fragments were then isolated on Denaturing Gradient Gel Electrophoresis (DGGE) to determine the bacteria and yeast communities.

3.3 DGGE analysis

Total composition of bacteria and yeast communities in sugary kefir grains and fermentates from sucrose media were investigated by PCR-DGGE analysis. After PCR fragments were isolated on a DGGE gel, DNA banding pattern of bacteria and yeast communities for kefir grains and fermentate were obtained (Fig. 2a-b). As shown in Fig. 2a, DNA bands of total bacteria amplified from kefir grain (GB) and fermentate (FB) contains six and five different bands, respectively. Bacterial DNA bands found in fermentate sample were less than those in kefir grains. There is one DNA band designated as GB3 which is found only in kefir grains.

For yeast communities, the DGGE fingerprints of the kefir grain (GY) and fermentate (FY) contain five and six different bands, respectively (Fig. 2b). There were three DNA fragments of kefir grains including GY1, GY4, and GY5 located at the same position as DNA fragments of fermentates FY1, FY5, and FY6, respectively. The other DNA fragments of kefir grains (GY2 and GY3) are at different locations from the fermentate DNA fragments (FY2, FY3, and FY4). The different positions of DNA fragments found in GY and FY suggested that some different yeast species were present in kefir grains and fermentates.

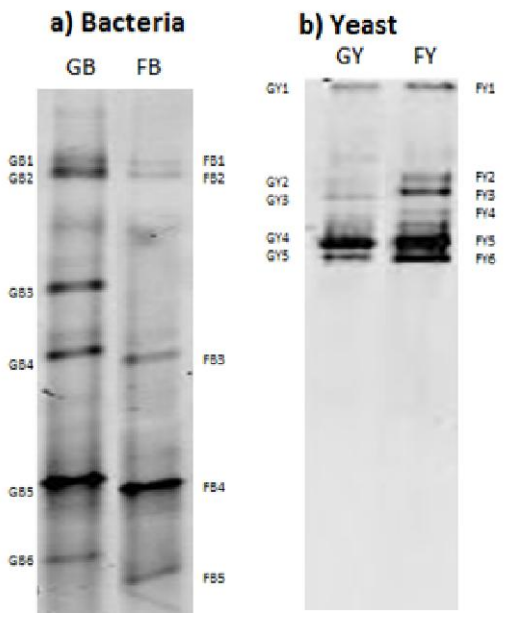

Fig. 2. Profiles of DGGE for 16S rDNA and 18S rDNA obtained from a) bacteria and b) yeast communities isolated from sucrose media; GB= bacteria in grain, FB= bacteria in fermentate, GY= yeast in grain, FY= yeast in fermentate.

3.4 Bacteria diversities of kefir grain and fermentate

Diversity of microorganisms present in sugary kefir grains and fermentates cultured in sucrose media were analyzed. Bacteria communities were determined from the obtained DGGE DNA sequences with the closest partial ribosomal DNA sequences reference in the BLAST database of GenBank. The bacterial communities in kefir grains and fermentates were identified with percent similarity as listed in Table 3. Among the DGGE bands of bacteria communities, two bacteria species including *Gluconobacter japonicus* and *Bacillus cereus* can be identified from the bands with a high similarity of 99% or more. Specifically, DNA bands GB4 and GB6 are identified as *Bacillus cereus* and *Gluconobacter japonicus*, respectively. DNA fragments FB3 and FB5, which are located at the same size as fragments GB4 and GB6, are also identified as *Bacillus cereus* and *Gluconobacter japonicus*, respectively.

In addition, possibly, there were three other bacteria including *Gluconobacter japonicus*, *Lactobacillus perolens*, and *Lactobacillus rhamnosus* which can be identified from the fragments GB2, GB3, and GB5, respectively. However, the closest similarities of GB2, GB3, and GB5 are less than 97%. In order to identify the microorganism species by comparing its DNA sequence with the available DNA in the database, the DNA similarity should be greater than 97% in sequence identity [25]. It should be noted that if the percent similarity is less than 97%, according to Stackebrandt [25], the two organisms may not be related at the species level. Nevertheless, DNA fragments FB1, FB2, and FB4 showed the closet similarity (less than 97%) to *Lactobacillus paracasei*, *Uncultured Bacilli bacterium*, and *Lactobacillus rhamnosus*, respectively. *Lactobacillus rhamnosus* was identified from both DNA fragments GB5 and FB4, which are located at the same position on DGGE gel. The DNA fragment GB3, which was found only in kefir grains, was identified as *Lactobacillus perolens* (less than 97% similarity). Surprisingly, GB2 and FB2, which are located at a similar position on DGGE gel, revealed different bacteria by the Blast similarity. The fragment GB1 could not be identified; however, the fragment FB1 with the same size of GB1 could be identified as *Lactobacillus paracasei* (less than 97% similarity). This phenomenon showed that two DGGE fragments with similar size could be identified as more than one microorganism [26].

Several studies on bacterial communities in sugary kefir reveal that the lactic acid bacteria genus *Lactobacillus* was commonly identified [13, 18, 24,27]. Acetic acid bacteria genus *Acetobacter* and *Gluconobacter* were rarely found [5-6]. In addition, *Bacillus* was also found in sugary kefir [9]. This study revealed the presence of the three genera of microorganisms which are commonly found in kefir beverage including *Lactobacillus perolens*, *Lactobacillus rhamnosus*, *Lactobacillus paracasei*, *Gluconobacter japonicus*, and *Bacillus cereus*. However, some of the identified microorganisms are found with the closest similarity of less than 97%.

Table 3 Identification of bacterial communiugary kefir grains and kefir fermentate

Sample	Accession NO.	Best match from Blast analysis	Fragment	% similarity
Grains	-	*Undefined bacterium*	GB1	-
	AB470922.2	*Gluconobacter japonicus*	GB2	97%
	AB690232.1	**Lactobacillus perolens*	GB3	90%
	KJ685393.1	*Bacillus cereus*	GB4	99%
	KM096587.1	**Lactobacillus rhamnosus*	GB5	89%
	AB470922.2	*Gluconobacter japonicus*	GB6	100%
Fermentate	AJ271854.1	*Lactobacillus paracasei*	FB1	90%
	EF703090.1	**Uncultured Bacilli bacterium*	FB2	94%
	HQ333012.1	*Bacillus cereus*	FB3	99%
	KM096587.1	**Lactobacillus rhamnosus*	FB4	89%
	AB470922.2	*Gluconobacter japonicus*	FB5	100%

*The percent similarities of these species are less than 97% as recommended by Stackebrandt [25] for microbial species assignment based on the 16s DNA homology.

Table 4 Identification of yeast communities in sugary kefir grains and kefir fermentate

Sample	Accession NO.	Best match from Blast analysis	Fragment	% similarity
Grains	EF176960.1	**Saccharomyces cerevisiae*	GY1	90%
	HM134859.1	**Saccharomyces cerevisiae*	GY2	89%
	LK021686.1	*Saccharomyces cerevisiae*	GY3	99%
	EF176960.1	**Saccharomyces cerevisiae*	GY4	90%
	JF274496.1	*Candida ethanolica*	GY5	99%
Fermentate	JQ698898.1	*Dekkera bruxellensis*	FY1	98%
	JF274496.1	*Candida ethanolica*	FY2	99%
	JF274496.1	*Candida ethanolica*	FY3	99%
	JF274496.1	*Candida ethanolica*	FY4	99%
	JF274496.1	*Candida ethanolica*	FY5	99%
	JF274496.1	*Candida ethanolica*	FY6	99%

*The percent similarities of these species are less than 97% as recommended by Stackebrandt [25] for microbial species assignment based on the 16s DNA homology.

3.5 Yeast diversities of kefir grain and fermentate

The yeast communities were identified according to 18S rDNA sequencing analyses. DNA sequences were determined with BLAST analysis of GenBank to obtain the yeast identities. The yeast species of sugary kefir grain and sugary kefir fermentate from the sucrose media are shown in Table 4. Two DNA fragments of kefir grain including GY3 and GY5 can be identified as *Saccharomyces cerevisiae* and *Candida ethanolica*, respectively with 99% similarity. *Candida ethanolica* was identified with 99% similarity to the five DNA fragments of fermentates (FY2, FY3, FY4, FY5, and FY6). This finding supported the phenomenon of DGGE method that one microorganism may be identified from more than one DNA fragment [28]. 18S rDNA is multiple alleles gene which can present multiple DNA fragments resulting in multiple DNA bands of one microorganism detected in DGGE analysis [29]. The fragments GY5 and FY6 which are located at the same position on DGGE gel were identified to be the same yeast species as *Candida ethanolica*.

Fragments of kefir grains GY1, GY2, and GY4 showed similar identity to *Saccharomyces cerevisiae*; however, the similarities were less than 97%. Moreover, fragment of fermentate FY1 showed 98% similarity to *Dekkera bruxellensis*. Although the fragments GY1 and FY1 are at a similar position on DGGE gel (Fig. 2b), the two fragments were not identified as the same microorganism.

Yeasts found in kefir were mostly from the genus *Saccharomyces* [10, 30], *Candida* [6, 11], *Kluyeromyces* [17], and *Pichia* [9, 18]. *Saccharomyces cerevisiae* is the major population found in kefir [8, 17, 31]. From the literature, the yeast genus *Candida* with different species, including *Candida kefyr, Candida maris, Candida krusei, Candida Iambica*, *Candida valdiviana*, and *Candida inconspicua,* were found in kefir [1, 4, 9, 11]. It is the first time to identify the yeast species *Candida ethanolica* in sugary kefir, by the present study. *Candida ethanolica* is a yeast that assimilates only ethanol as a carbon source without requiring nitrate or urea as nitrogen source [32]. In addition, this yeast can grow at high temperature (35 – 37 °C) [33]. Therefore, the culture conditions of sugary kefir used in this study was preferable for the growth of *Candida ethanolica*.

From the literature, there are a few studies on yeast species *Candida ethanolica.* It was first separated from the fodder yeast industry [32]. *Candida ethanolica* was also isolated from fermented pineapple. The yeast species was also studied for an agricultural purpose as pest control of snails in rice cultivation [34]. Thus, the by-product generated from the sugary kefir culture that contains *Candida ethanolica* may possibly be used as a biological control agent in agricultural applications.

This study shows that the bacteria and yeasts presented in sugary kefir grain and fermentate including *Bacillus cereus, Lactobacillus paracasei*, *Lactobacillus rhamnosus*, and *Saccharomyces*

cerevisiae are considered to be probiotic microorganisms [35-37]. Therefore, the Thai sugary kefir drink studied in the present work may be beneficial for human health. In addition, the information concerning the microorganisms species present in the Thai sugary kefir may be used as guideline for sugary kefir drink production.

3.6 Microbial morphology in kefir grain by SEM Analysis

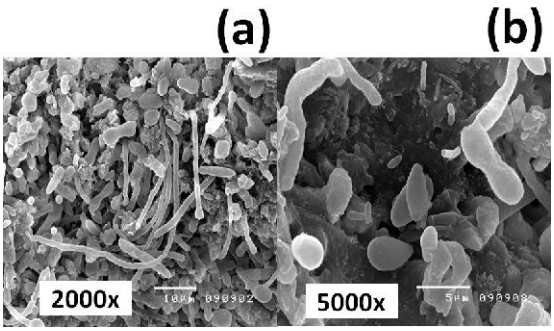

Fig. 3. Bacteria and yeast distribution in kefir grain on the (a) outer surface and (b) inner surface

The possible microorganism compositions present in kefir grains which were cultured in the sucrose media were examined by SEM analyses (see Fig. 3). The sugary kefir grains cultured for 3 days were imaged for microbial distribution. The SEM images revealed that microbial communities of the sugary kefir grain consisted of both bacteria and yeasts. Fig. 3a (2,000X) shows yeast communities located on the outer surface of the grain. There are three characteristics of yeasts including circular, lemon, and elongated shapes found on the grain outer surface. The microbial morphology observed by SEM suggested that the circular shape yeasts may be *Dekkera bruxellensis*, and *Saccharomyces cerevisiae,* which are well-known yeasts with lemon shape. Fig. 3b shows the inner surface of the kefir grain at 5,000X magnification. The inner surface of the grain hosts bacterial communities growing in kefir grain. Three bacterial shapes including cocci, small bacilli, and big bacilli were observed on the inner surface. The cocci and small bacilli characters could belong to acetic acid bacteria and lactic acid bacteria, respectively. The big bacilli could be *Bacillus cereus* because the bacteria identified from DGGE fragment is a big bacilli bacteria [38]. Acetic acid bacteria identified in this study are *Gluconobacter japonicus*, and lactic acid bacteria include regular *Lactobacillus perolens*, *Lactobacillus rhamnosus*, and *Lactobacillus paracasei*. The regular distribution of the bacteria and yeasts in the kefir grains, where the yeasts community formed on the outer surface of kefir grains and the bacteria grew adjacently, implies symbiotic relationships among the microorganisms found in the kefir grains [39-40]. The interaction among microorganisms found in kefir grain is considered as mutualism. Yeasts growing in the kefir grain released essential nutrients for bacteria in the preference condition which is stimulated by bacteria. However, this phenomenon only occurs in co-cultivation.

4. Conclusions

The microbial communities present in sugary kefir grains and sugary kefir fermentate cultured in sucrose were analyzed by PCR-DGGE analysis. Sugary kefir grains and fermentates have higher diversity of bacteria communities than yeast communities. Acetic acid bacteria *Gluconobacter japonicus* and *Bacillus cereus* were identified with 100% and 99% similarity. Other bacteria species including *Lactobacillus rhamnosus* were also identified with less than 97% similarity. Two yeast species, namely *Saccharomyces cerevisiae*, *Candida ethanolica*, were identified with 99%. It was the first time that the yeast *Candida ethanolica* was found in sugary kefir. Microbial communities of sugary kefir grains consist of bacteria and yeast. The yeast communities were located on the outer surface of the kefir grains, while the bacterial communities were located on the inner surface of the kefir grains.

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