Asian Jr. of Microbiol. Biotech. Env. Sc. Vol. 22, No. (1) : 2020 : 44-49

ISSN-0972-3005

LACTIC ACID BACTERIA AND YEASTS FROM INDONESIAN KEFIR GRAINS AND THEIR GROWTH INTERACTION

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(Received 28 October, 2019; accepted 30 November, 2019)

Keywords: *Co-culture, Kefir grain, Lactic acid bacteria, Lactobacillus, Yeasts.*

Abstract – Kefir grain, a starter culture for kefir fermentation, is polysaccharides and protein matrixes consisting of various lactic acid bacteria and yeasts. The aim of this study was to (1) enumerate, isolate, and identify predominant LAB and yeast from Indonesian kefir grains and (2) evaluate the growth of LAB and yeast isolates in skim milk. The results revealed that the LAB counts reached 9 log CFU/g, while the yeast counts reached 8 log CFU/g. Presumptive *Lactobacillus* reached 8 log CFU/g, which was higher than *Lactococcus* and *Leuconostoc*. Identification by API test kits revealed that the predominant species of LAB were *Lactobacillus fermentum 1, Lactobacillus brevis 3, Lactobacillus paracasei ssp. paracasei,* and *Lactococcus lactis ssp. lactis*. The predominant yeasts were *Saccharomyces cerevisiae, Candida krusei/inconspicua, Candida parasilopsis, Candida glabrata,* and *Kloeckera spp.* The ability of LAB and yeast isolates to acidify and coagulate milk varied. Co-culture of *Lactobacillus rhamnosus* YK12 and *Saccharomyces cerevisiae* YKPD6 showed that both were growing well in skim milk, suggested that both isolates could be used to develop a defined starter culture for kefir fermentation.

INTRODUCTION

Kefir is a fermented milk beverage with unique flavors. The flavors are slightly sour, uniform creamy, carbonated, and slightly alcoholic (Farnworth, 2005). Kefir is traditionally produced using a starter called kefir grain or kefiran (Moradi and Kalanpour, 2019). The grains are composed of polysaccharides and proteins (Hamet *et al*., 2015). Both matrixes are produced by certain microbes when kefir fermentation occurs. Previous studies reported that the grains are dominated by different species of LAB and yeasts (Gao and Li, 2016). The difference of predominant species in the grains are related to the grains origin and handling process (Nalbantoglu *et al*., 2014; Gao and Li, 2016). However, there are several species of LAB and yeasts that had been consistently identified, namely *Lactobacillus kefiri* (*Lb. kefiri*), *Lb.kefiranofaciens, Lactococcus lactis* (*Lc. lactis*), *Streptococcus thermophilus, Leuconostoc mesenteroides* and *Saccharomyces cerevisiae* (*S. cerevisiae*) (Kesmen and Kacmaz, 2011; Hsieh *et al*., 2012; Leite *et al*., 2012; Lima *et al*., 2017).

The complex microbiological association in kefir grains makes difficult to obtain defined and constant kefir starter culture appropriate for industrial kefir production of conventional properties (Nambou *et al*., 2014; Walsh *et al*., 2016). The strains isolated from kefir grain sometimes cannot be reproduced, and metabolize optimally (Cheirsilp *et al*., 2003; Stadie *et al*., 2013). Therefore, analysis of fermented products by the strains isolated from kefir grains is needed.

Kefir is well known in Indonesia, although it is not as popular as yogurt. Kefir grains used in several regions in Indonesia, are thought to contain different predominant LAB and yeasts. The aim of this study was to (1) enumerate, isolate, and identify predominant LAB and yeast from Indonesian kefir grains and (2) evaluate the growth of LAB and yeast isolates on skim milk.

MATERIALS AND METHODS

Kefir Grains

Kefir grains are obtained from household kefir

producers in Bogor (BG), Bandung (BD), Jakarta (JK), and Yogyakarta (YK). The condition of the grains when shipping was semi-wet, put in a cool box with a temperature of 4-10 °C and stored in the refrigerator before analyzed. The kefir grains were activated by adding 50g of grains to 500 mL of sterilized milk, followed by incubation at 27 °C for 24 h. The grains were retrieved by sieving, reinoculated into fresh milk, and incubated at 27 °C for 24 h. This step was repeated 3 times. After this procedure the grains were considered active and ready to be analyzed.

Enumeration, Isolation and Characterization of Predominant Lactic Acid Bacteria and Yeasts

The method for enumeration of predominant LAB and yeast refers to the research conducted by Garofalo (Garofalo *et al.*, 2015). As much as 25g of each of the kefir grains were diluted with 225 mL of KH_2PO_4 solution and then transferred into Stomacher (Bag Mixer; Interscience, FRA) for 10 min. Serial decimal dilutions were prepared by using 10 mL of each of the homogenized suspensions of kefir grains. Total LAB were enumerated on De Mann Rogosa Sharpe agar (MRSA; Oxoid, USA) incubated in anaerobic condition (Anoxomat AN2CTS, Mart Microbiology BV, NED) at 37 °C for 48 h. *Lactobacilli* were enumerated on Rogosa agar (RA; Merck, GER), incubated in anaerobic condition at 37 °C and 42 °C for 48 h. Presumptive *Lactococci* and *Leuconostoc* were enumerated on M17 agar (Merck, GER) and MRSA+vancomycin (200ppm; Oxoid, USA) respectively, incubated at in anaerobic condition 37 °C for 48 h. In order to inhibit yeast growth, 200 ppm cycloheximide (Sigma, USA) was added. Total yeasts were enumerated on Potato Dextrose agar (PDA; Oxoid, USA) supplemented 200 ppm chloramphenicol (Sigma, USA) to inhibit bacteria growth, incubated at 25 °C for 72 h.

After the incubation period, the plates containing between 30 and 300 colonies were selected for enumeration. From each of the different observed colony types, one colony was selected and streaked on new plates in order to obtain pure colonies.The LAB colonies were subjected to Gram staining and the catalase test (Coico, 2005). The yeast colonies were subjected to morphology examination, urease test (Kurtzman *et al.*, 2011), and fermentation toward glucose, fructose, and sucrose (Kurtzman *et al.*, 2011). The pure isolates of LAB and yeast were stored as frozen stock at –20 °C in MRS and PD broth with 20% glycerol.

Identification of LAB and yeast isolates by Analytical Profile Index (API)

LAB and yeast isolates were identified with API 50C HL kit (Bio-Merieux, FRA) and API 20C AUX kit (Bio-Merieux, FRA), respectively. The identification was done according to the manufacturer instruction.

The growth of LAB and yeasts isolates in skim milk

Growth testing in skim milk was carried out on three isolates of LAB and three isolates of yeast from four grains, which were different species. Each isolate was inoculated as much as 2% (v/v) into sterile skim milk (12%; w/v), then incubated at 37 °C for 48 h. Observations were made on the pH value (pH meter; Eutech pH 700, USA), the amount of whey, coagulant texture, and aroma.

Co-culture of LAB and yeast isolates in skim milk

Referring to the results of growth test in skim milk, each of isolate of LAB and yeast were selected. Selected LAB and yeast culture were inoculated (2%; v/v) as co-culture into sterile skim milk and incubated at 37 °C for 48 h. Observations were made on the pH value, the number of colonies (Boczek *et al.*, 2014), total acid (Tyl and Sadler, 2017), and ethanol concentration (Nambou *et al.*, 2014).

RESULTS AND DISCUSSION

Enumeration of predominant LAB and yeasts

The results of enumeration showed the total LAB ranged from 1.1± 0.7 $x10^8$ to 1.1 ± 0.2 $x10^9$ CFU/g, while the total yeasts ranged from 2.3± 0.9 $x10^6$ to 6.5 ± 4.2 $x10^6$ CFU/g (Table 1). Enumeration on selective media showed that presumptive mesophilic *Lactobacillus* was the most dominant compared to other LAB groups, ranged from 1.9± 1.0 $x10^8$ to 1.4 ± 0.3 $x10^9$ CFU/g. The results of this enumeration also confirm the results of previous studies that showed *Lactobacilli* was more dominant than *Leuconostoc* and *Lactococcus* (Garofalo *et al.*, 2015; Wang *et al.*, 2015). This high number of LAB and yeasts indicating that kefir grains were a comfortable and beneficial ecosystem for LAB and yeasts. Kefir grains are thought to be potential sources of LAB and yeasts. Many studies have reported that LAB and yeasts were a group of microorganisms that can meet the probiotic criteria (Gil-Rodríguez *et al.*, 2015; Zeng *et al.*, 2016) and have been widely used in the food

Table 1. LAB and yeast counts carried out on four Indonesian kefir grains

Grains	Total LAB (CFU/g)	Total yeast (CFU/g)	Total colony (CFU/g)			
			Lactobacilli termofilik	*Lactobacilli mesofilik*	*Leuconostoc*	*Lactococci*
BG	1.1±0.2 x10^{9a}	6.2± 8.3 x10^{7a}	5.3± 7.5 x10^{6a}	1.9± 1.0 x10^{8a}	2.5± 0.0 x10^{1a}	2.3± 1.5 x10^{2a}
BD	1.1±0.2 x10^{9a}	4.4± 4.0 x10^{6a}	1.7± 2.4 x10^{6a}	1.4± 0.3 x10^{9b}	2.5± 0.0 x10^{1a}	1.2± 1.3 x10^{2a}
JK	1.1± 0.7 x10^{8a}	2.3± 0.9 x10^{6a}	8.3± 12 x10^{7a}	3.3± 2.2 x10^{8a}	1.1± 1.2 x10^{2a}	2.8± 2.2 x10^{2a}
YK	8.3± 3.0 x10^{8a}	6.5± 4.2 x10^{6a}	2.9± 4.1 x10^{8a}	7.6± 2.7 x10^{8a}	4.0± 2.8 x10^{2b}	2.0± 2.5 x10^{2a}

Means ± standard deviations of duplicate independent experiments are shown. Means followed by different letters are significantly different (P < 0.05).

industry (Nielsen *et al*., 2014).

Isolation and preliminary identification of predominant LAB and yeasts

Sixty LAB and forty yeast colonies from four Indonesian kefir grains, were isolated and purified. All LAB isolates were Gram-positive and catalase-negative. Four isolates were round shape while the rest were rod shape. As for yeast isolates, the cells were rod, oval, or round shapes. All yeast isolates were urease-negative and fermentation-positive to glucose, fructose and sucrose (Table 2). Previous studies reported that many species of LAB and yeast with varying characteristics were isolated from kefir grain (Garofalo *et al*., 2015). Conventional morphological and biochemical characterization were important as preliminary test for grouping of isolates.

Identification of isolates by analytical profile index (API)

Based on their morphology and preliminary identification, thirty LAB isolates and thirty yeast isolates were selected to be identified. The identification showed that each Indonesian kefir grain was dominated by various species (Figure 1). BG grain was dominated by *Lb. fermentum* 1. BD grain was the most diverse grain, which inhabited *Lc. lactis ssp. lactis, Lb. brevis 3, Lb. paracasei ssp. paracasei, Lb. plantarum 1,* and *Lb. delbrueckii ssp. delbrueckii.* Species *Lb. fermentum* 1 was found in throughout grains, while *Lb. delbrueckii ssp. delbrueckii* specific only in BD grain and *Lb. rhamnosus* specific only in YK grain. Identification of yeast showed that *S. cerevisiae* and *Candida krusei/inconspicua* (*C. krusei/inconspicua*) were dominant (Figure 2). *S. cerevisiae* was identified throughout grains. There were several yeasts that were identified only in one grain, namely *C. parasilopsis* on BG grain, *C. glabrata* on BD grain, and *Kloeckera spp*. on YK grain.

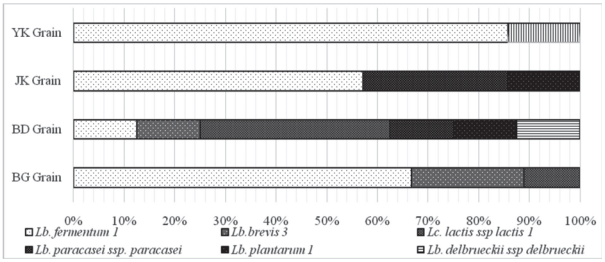

Fig. 1. Composition of predominant species of LAB in four Indonesian kefir grains.

It was suspected that the predominant LAB and

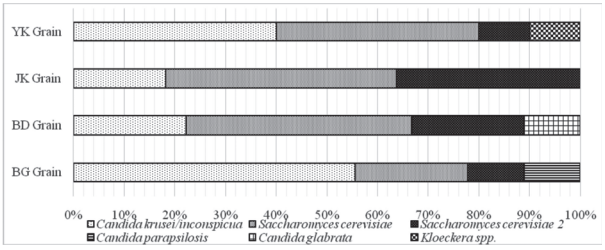

Fig. 2. Composition of predominant species of yeasts in four Indonesian kefir grains.

Table 2. Number of LAB and yeasts isolated from Indonesian kefir grains based on characterization results.

Grains	Number isolate of LAB		Number isolate of Yeasts		
	Bacil, Gram (+) Catalase (-)	Coccus, Gram (+) Catalase (-)	Growth in Glu (+), Fru (+), Suc (+), Urea (-)		
			Oval	Coccus	Bacil
BG	14	1	4	3	3
BD	12	3	3	1	6
JK	15	-	2	3	5
YK	15	-	1	2	7

yeasts may be attributed to the increasing acidity of the fermenting kefir. Different microbial composition thought due to competitive between the microorganisms and the inability to grow at lower pH values (Liu *et al.*, 2015). LAB and yeasts identified in Indonesian kefir grains, have been identified in the grain from various countries (Gulitz *et al.*, 2011; Huang *et al.*, 2013). And have known to be non-pathogenic and generally present in fermented foods. Likewise, *S. cerevisiae* was widely used in the food industry. *C. krusei/inconspicua* was lived in fruits or soils, that was rarely causing the outbreaks (Parmeland *et al.*, 2013). Similarly, *C. parasilopsis, C. glabrata,* and *Kloeckera sp.*were opportunistic-pathogenic for patients with immune problems (Trofa *et al.,* 2008; Rodrigues *et al.,* 2014).

Growth of LAB isolates in skim milk

The results showed that LAB isolates were able to acidify skim milk to pH 3.9-4.7 after 48 h at 37 °C (Table 3). Some isolates were formed the compact coagulants and whey. The production of CO_2 was thought to cause separate coagulants. *Lb. paracasei, Lb. fermentum* and *Lb. brevis* were heterofermentative that capable to produce CO_2 (Hayward, 1957).

Growth of yeast isolates in skim milk

The results showed that yeast isolates cannot coagulate skim milk as performed by LAB. However, the aroma of skim milk was more refreshing. The yeasts were reduced to pH 5.1 and produced weak and separate coagulant (Table 3). All yeast isolates were able to produce slightly sour aroma, mixed with the aroma of alcohol. Various and better aroma of combined sour milk with alcohol and fruit aroma was produced by *S. cerevisiae* YKPD6. It was reported that in kefir fermentation, yeasts were consumed some carbon sources, like glucose and lactose (Cheirsilp *et al.*, 2003a) then produced several esters, aldehydes, and ketones (Nambou *et al.*, 2014).

Co-culture between LAB and yeast isolates in skim milk

Based on observations on the number of colonies,

Table 3. LAB and yeasts isolated from Indonesia kefir grains profiles on skim milk at 37°C for 24 h.

Grains	Name of isolates	Whey	pH	Coagulant	Aromatic scent
BG	*Lb. fermentum* BG 5	++++	4.5	Weak	Sour
	Lb. fermentum BG 8	++	4.2	Compact	Sour
	Lc. lactis ssp lactis BG M17 2	+++	4.1	Separate	Sour
	C. krusei/inconspicua BG PD1	++++	5.9	Weak	Sour+alcohol
	S. cerevisiae BG PD2	+++	5.3	Weak	Sour+alcohol
	C. parapsilosis BG PD8	+++++	5.7	n.d	Sour+alcohol
BD	*Lb. paracasei ssp. paracasei* BD2	++	3.9	Compact	Sour
	Lc. lactis ssp lactis BD 3	++	3.7	Compact	Sour
	Lb. plantarum BD 4	++	4.7	Weak	Sour
	Lb. brevis BD 5	+++++	4.3	Separate	Sour
	Lb. fermentum BD 6	+	4.1	Compact	Sour
	Lb. delbrueckiisspdelbrueckii BD7	+++++	4.5	Separate	Sour
	S. cerevisiae BD PD1	++	5.2	Weak	Sour+alcohol
	C. glabrata BD PD5	++++	5.8	Weak	Sour+alcohol
	C. krusei/inconspicua BD PD7	+++++	6.1	n.d	Sour+alcohol
JK	*Lb. fermentum* JK 1	++++	3.7	Separate	Sour
	Lb. paracasei ssp. paracasei JK13	++++	3.9	Separate	Sour
	Lb. fermentum JK 17	+++	3.8	Weak	Sour
	S. cerevisiae JK PD4	++	6	Weak	Sour+alcohol
	C. krusei/inconspicua JK PD6	+++++	5.1	n.d	Sour+alcohol
	C. krusei/inconspicua JK PD3	+++	5.3	Separate	Sour+alcohol
YK	*Lb. fermentum* YK 4	++	4.1	Weak	Sour
	Lb. fermentum YK 7	+++	4.6	Separate	Sour
	Lb. rhamnosus YK 12	+	3.8	Compact	Sour
	C. krusei/inconspicua YK PD2	+++++	5.2	n.d	Sour+alcohol
	S. cerevisiae YK PD6	++	5.7	Weak	Sour+fruit+alcohol
	Kloeckera spp. YK PD7	++	5.7	Separate	Sour+alcohol

n.d = not detected; +++++ = 10 ml; ++++ = 7.5 ml; +++ = 5 ml; ++ = 2.5 ml; + = < 2.5 ml.

Table 4. Profiles of co-culture *Lb. rhamnosus* YK12 and *S. cerevisiae* YKPD6 isolated from Indonesia kefir grains on skim milk at 37 °C for 24 h.

No	Atributes	Incubation (Hours)	*Lb. rhamnosus* YK12	*S. cerevisiae* YKPD6	Co-culture *Lb. rhamnosus* YK12 & *S. cerevisiae* YKPD6	
1	Colony count	0	5.92 [a]	5.40 [a]	LAB: 6.00 [a]	Yeast: 5.40 [a]
	(log CFU/ml)	4	5.33 [b]	6.33 [b]	LAB: 6.07 [b]	Yeast: 5.24 [b]
		8	6.10 [b]	6.99 [b]	LAB: 6.74 [b]	Yeast: 6.87 [b]
		24	8.48 [c]	7.24 [b]	LAB: 8.22 [d]	Yeast: 7.48 [b]
		48	9.08 [c]	7.64 [b]	LAB: 9.02 [cd]	Yeast: 8.01 [b]
2	pH	0	6.36 [c]	6.47 [c]	6.78 [d]	
		4	6.25 [c]	6.31 [bc]	6.18 [cd]	
		8	6.24 [c]	6.29 [bc]	6.11 [c]	
		24	4.37 [b]	5.97 [b]	4.85 [b]	
		48	3.88 [a]	5.34 [a]	3.87 [a]	
3	Total acid (%)	0	0.09 [a]	0.08 [a]	0.06 [a]	
		4	0.3 [b]	0.27 [b]	0.3 [b]	
		8	0.32 [b]	0.32 [b]	0.41 [b]	
		24	0.65 [b]	0.6 [bc]	0.76 [c]	
		48	1.52 [c]	0.82 [c]	1.46 [d]	
4	Alcohol (mg/l)	24	< 2.8	3.1	2.42	
		48	< 2.8	3.27	3.58	

Means followed by different letters are significantly different ($P < 0.05$).

pH value, total acid, and ethanol concentration after 48 h of incubation in Table 4, showed that *Lb. rhamnosus YK12* and *S. cerevisiae YKPD6* were capable to grow in co-culture, without being affected by other's existence. No-intrusive interactions between LAB and yeast strain in a fermentation system, have previously been reported (Cheirsilp *et al.*, 2003b). Further research was still needed to determine the mutualism symbiosis that would occur.

Traditional kefir have some profiles that pH in the range 4.3-4.4, lactic acid around 0.5 - 0.8%, and alcohol around 0.2% (Magalhães *et al.*, 2011; Nambou *et al.*, 2014). In general, these profiles were similar to fermented milk produced by the co-culture in this study. It can be presumed that *Lb. rhamnosus* YK12 and *S. cerevisiae* YKPD6 could be used as starter culture to defined starter culture for fermentation of kefir.

Indonesian kefir grains are dominated by various species of LAB and yeasts. The grains can be an alternative source of LAB and yeasts. Co-culture among *Lb. rhamnosus* YK12, which good for coagulating milk, and *S. cerevisiae* YKPD6 which good for aroma production, has shown that both cultures could grow well in skim milk. The pH, lactic acid and ethanol content of fermented milk produced by the co-culture shows similarities to traditional Kefir that was reported.It can be presumed that *Lb. rhamnosus* YK12 and *S. cerevisiae* YKPD6 could be used as defined starter culture to produce kefir. Further research is needed to characterize the resulted kefir and evaluate its functional properties.

ACKNOWLEDGMENT

This research was supported by the endowment fund for education (LPDP), from the Ministry of Finance Republic for the recipient Dandy Yusuf.

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