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# Short Communication: Diversity of indigenous LAB from kefir grains cultured in goat milk based on phenotypic characteristics for probiotic candidates

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**Abstract.** *Wulansari PD, Rahayu N, Kusuma RJ, Sukarno AS. 2023. Short Communication: Diversity of indigenous LAB from kefir grains cultured in goat milk based on phenotypic characteristics for probiotic candidates. Biodiversitas 24: 6389-6395.* Kefir grain is the starter of kefir products containing the symbiotic compound of lactic acid bacteria (LAB) and yeast. Microbial diversity in kefir grain is affected by multiple factors that include the origin of microbiota, upkeep, storage, and types of milk. Goat milk exhibits various superior properties in composition or characteristics. These have inspired the present study to obtain the local isolates from LAB derived from native Indonesian kefir grain produced and revitalized using goat milk. This study aimed to obtain local LAB isolates from kefir grain through phenotypic identification and characterization. The morphology of phenotypic identification consisted of macroscopic, microscopic, and biochemical identification. Based on macroscopic and microscopic observation, the isolates were milky white, circular, entire edge, smooth and shiny surface with convex elevation, coccus/basil shaped, Gram-positive, negative catalase, non-motile, non-spore, non-producing $CO_2$, heterofermentative/homofermentative, able to ferment various carbohydrates, growing at 37 and 45°C, pH 4.4, and salinity level of 6.5%. A scientific approach enabled to obtaining of several LAB strains, namely nine isolates of *Lactococcus* and seven isolates of *Lactobacillus*. It takes genotypic identification to promote the identification process up to the species level.

**Keywords:** Biochemical identification, isolates, LAB, phenotypic identification, probiotics

## INTRODUCTION

Consumers' awareness of healthy diet and wellness has inspired the development of probiotic-loaded functional food. Demand for probiotic functional food has increased and boosted the food industry (Hennessy 2014). Probiotic is defined as a living microorganism which, when consumed at a particular amount, gives health benefits beyond its inherent nutrition (Wang et al. 2015). The main group in probiotic bacteria is lactic acid bacteria (LAB). LAB are considered probiotics if they can withstand acidic environments and bile salt exposure in the body, as well as exhibit good intestinal absorbability and good correlation with health signs in clinical tests (Champagne et al. 2018).

The commercial availability of LAB as a probiotic strain is prevalent around the globe, but screening for a new strain is attractive to industry (Ayeni et al. 2011). The first screening of LAB strains was isolated from milk, and since then, other LAB strains have been discovered in cow milk and dairy products (Taye et al. 2021), fermented products (Vasyliuk et al. 2014), fermented drinks (Susan et al. 2020), and also kefir (Zanirati et al. 2015; Yerlikaya 2019; Plessas et al. 2020). Milk or dairy products (fermented milk) are the source of good probiotics included in the Generally Recognized as Safe (GRAS) group. Kefir is a probiotic source because it can improve health and nutritional status. As one of the fermented drinks, kefir tastes a little sour and mildly alcoholic, is easily digestible, and has been a popular consumed product (Sharifi et al. 2017). Kefir is produced from fermentation using kefir grain containing symbiotic complex LAB and yeast embedded in exopolysaccharide matrices (Yovanoudi et al. 2013; Zanirati et al. 2015). Several LABs in kefir grains exhibit probiotic characteristics (Ganatsios et al. 2021). Isolates from traditional fermented dairy products can be used as probiotic agents that should be conserved, added, and incorporated into food products (Nemati et al. 2023).

Isolating LAB as the probiotic candidate derived from kefir grain has been performed across the globe, such as in Brazil (Leite et al. 2015; Zanirati et al. 2015), China (Rajoka et al. 2019), Malaysia (Talib et al. 2019), Turkey (Yerlikaya 2019), Indonesia (Yusuf et al. 2021) and many others. While studies on LAB isolated from kefir grains are plenty, there have been few investigations on the structure and microbial diversity of kefir grain and the multivarious contributing factors that include microbiota, maintenance and storage condition, microbiological composition, production process, time and temperature of fermentation,

and types of milk (Garofalo et al. 2015; Schwan et al. 2016; Ma'mon et al. 2018). Previous studies on bacterial communities have confirmed that kefir grain produced in different locations with different methods shows microbiota diversity (Marsh et al. 2013; Nalbantoglu et al. 2014).

The microbiota in milk has a direct effect on its quality, safety, and durability, with some strains reported to exhibit industrial benefits (*Saccharomyces, Lactococcus,* and *Kluyveromyces*), it should be healthy-promoting characteristics (*Bifidobacterium, Weissella,* and *Lactobacillus*), health risks (*Brucella, Clostridium,* and *Escherichia coli*), or food spoilage (*Kurthia, Micrococcus,* and *Streptococcus*) (Oikonomou et al. 2020; Akinyemi et al. 2021). Results of the metagenomic analysis indicate that types of milk (goat milk vs. cow milk) affect microbial or yeast diversity. A previous study has predicted the functional properties of microbial composition kefir grains from these two types of milk and reported that kefir grain from goat milk contains more amino acid metabolism, carbohydrate, energy, and cofactor; biosynthesis and glycan metabolism; and vitamin than kefir grain from cow milk (Sumarmono et al. 2023). This is probably because goat milk has superior nutritional properties, such as fat, protein, ash, vitamins, lactose, and enzymes, compared to other milk and other functional properties (Getaneh et al. 2016). Accordingly, a closer examination of the composition and potential of LAB derived from goat milk kefir grain is enticing to obtain new isolates as the potential of probiotic LAB. This study aimed to undertake phenotypic identification and characterization of LAB isolated from kefir grain cultured in goat milk.

## MATERIALS AND METHODS

### Sample collection

This study used kefir grain revitalized in goat milk as the sample. Commercial kefir grains in this study were derived from Kefira (Yogyakarta, Indonesia). Exactly 50 g kefir grain was inoculated into 500 mL pasteurized goat milk and then incubated at 25°C for three days. This fermentation process was repeated several times until the kefir grain exhibited the desired characteristics and biomass increased by approximately 10% (Nalbantoglu et al. 2014).

### Enrichment and dilution of sample

Sample enrichment and dilution were conducted using a method by Anindita (2022), while kefir grain was enriched, both aerobic and anaerobic, using de Man Rogosa and Sharpe Broth (MRSB) (Merck, Germany). Anaerobic enrichment was conducted by incorporating 10 g of sample into 90 mL MRSB (Merck, Germany) + 0.15% bile salt (Oxoid, United Kingdom) + 50 ppm anti-yeast (Solinfeca, Indonesia). The aerobic enrichment mixed 10 g kefir grain sample with 90 mL MRSB + 0.15% bile salt + 0.05% L-cysteine (Merck, Germany) + 50 ppm anti-yeast. Both samples were incubated at 37°C for 18 hours. The incubation yield was collected (100 µL) and added with 900 µL peptone water to obtain $10^{-1}$ dilution, then diluted again until $10^{-9}$.

### Isolation of probiotic LAB

Isolation of probiotic LAB was undertaken by modifying a method by Ismail et al. (2018). The purification was performed by collecting 1 $10^{-9}$ samples using a round inoculating loop, then inoculating it into de Man Rogosa and Sharpe Agar (MRSA) (Merck, Germany) + 0.15% bile salt by making a streak in a zig-zag motion on a Petri plate to create four quadrants. The incubation was performed at 37°C for 24 to 48 hours. The purification was replicated four times to obtain perfectly uniform isolates. Then, colonies growing on the plate were isolated based on their appearance. One colony was collected from each plate to be inoculated into the MRSB media at 37°C for 18 hours. This procedure was repeated until a single isolate or colony was obtained from each petri dish.

### Phenotypic identification based on morphology

The morphological properties of LAB were subjected to macroscopic and microscopic observation (Yerlikaya 2019). The macroscopic observation was performed without equipment to observe the color, shape, perimeter, surface, elevation, and spore. Microscopic observation uses a microscope (Celestron, US) with 1,000x magnification to observe the cell shape and structures.

### Phenotypic identification based on biochemistry

**Gram staining**. Gram staining was performed on bacterial culture grown in an MRSA medium for 24 hours (Romadhon and Margino 2012; Detha 2019; Welsh 2019). Isolates of MRSA were collected using a loop, then stained on an object glass, and fixated on a Bunsen burner. The cells on the preparation were stained with violet crystal solution (Gram A) (Himedia, India) for 60 seconds, rinsed under running water, and then dried. After this, the preparation was stained with iodine solution (Gram B) (Himedia, India), left for two minutes, rinsed under running water, and dried. In the next step, the preparation was stained with 96% alcohol (Gram C) (Himedia, India) until the purple color disappeared. Finally, the preparation was stained with safranin (Gram D) (Himedia, India), let sit for 30 seconds, rinsed under running water, and dried. The preparate was put under a microscope to observe the gram staining results and lactic acid bacteria's cell shape and structures. LAB are gram-positive bacteria, so purple is expected to appear in the gram-staining test.

**Catalase test**. One loop of an isolate from bacteria culture aged 24 hours was stained on a glass objected and incorporated with two drops of $H_2O_2$ 3% (Merck, Germany), then observed for a gas bubble formed on the glass to measure the catalase activities (Mulaw et al. 2019). The gas bubble shows a positive reaction of catalase tests, indicating the formation of oxygen ($O_2$) by the catalase enzyme in the bacteria when degrading $H_2O_2$. Meanwhile, in the negative reaction, no gas bubble is formed.

**Motility test.** The isolates of MRSA media were inserted into a semi-solid straight agar (upright SIM media) and then let sit for incubation at 37°C for 48 hours to test

the motility of LAB (Detha 2019; Zhi et al. 2021). When patches were formed around the area where the needle was punctured on the medium, It showed a positive result (motile); otherwise, it was negative (non-motile).

**Types of fermentation.** The fermentation test was conducted by growing a bacteria culture on an MRSB inside a test tube filled with a Durham tube (Romadhon and Margino 2012). Incubation was conducted for two days while observing the formation of gas bubbles in the Durham tube; the fermentation test aimed to categorize the LAB into either homofermentative or heterofermentative groups.

**Fermentation of carbohydrates.** A 5 gr/L carbon source was mixed with MRSB and incorporated into a test tube filled with an 8-mL Durham tube. Into the test tube were added 24-hour isolates, then incubated at 37°C for one day. The source of carbons for carbohydrate fermentation tests was glucose, D-galactose, glycerol, D-fructose, D-sorbitol, D-lactose, and D-saccharose. The bacterial growth was observed from its turbidity using a spectrophotometer at 620 nm with an optical density (Zhi et al. 2021).

**Physiology of different temperature, pH, and salt concentration**

The growth of LAB isolates at different temperatures, pH, and salt concentrations in the present study aimed to analyze the physiological characteristics of the (Romadhon and Margino 2012; Anindita 2022).

**Different temperatures:** the effect of different temperatures on the growth of bacteriocin-producing isolates was analyzed by incubating bacteria in an MRSB at 10, 37, and 45°C for 24 hours. Bacterial growth was marked by turbidity in the MRSB media.

**Different pH:** the effect of pH on the isolates was observed by growing the isolates in an MRSB with a pH of 4.4. and 9.6, added with 1 N NaOH (Merck, Germany) or 1 N HCL (Merck, Germany), then incubated for 24 hours at 37°C. Likewise, bacterial growth was marked by turbidity formed in MRSB.

**Salt concentration:** To analyze the effect of bacteriocin-producing isolates on salt concentration by growing the isolates in an MRSB with 6.5 and 18% salt concentration for 24 hours. Any turbidity shown in the isolates was indicative of bacterial growth. This series of isolation and identification of goat-milk kefir grain are presented on the research flowchart in Figure 1.

**Data analysis**

The obtained data were subjected to a descriptive qualitative method.

## RESULTS AND DISCUSSION

**Phenotypic identification with a basis of morphology**

Moreover, 16 isolates were obtained from the results of isolating goat-milk kefir grain, then characterized and identified up to genus level using the phenotypic method. Generally, the phenotypic method to identify the characteristics of new LAB isolates relies on the physiological or biochemical criteria (Kongo et al. 2007). The macroscopic and microscopic criteria of isolates are presented in Table 1. The results of macroscopic identification showed that the isolates were milky white and circular, had an intact perimeter, smooth and shiny surface, and convex elevation. The microscopic morphological characteristics showed that the cells were both cocci (spherical shape, n=9) and bacilli (rod shape, n=7). Based on this data, the selected isolates were most likely from the LAB group because of their resemblance with those of LAB. The colony of LAB is spherical, white to yellowish colored, and elliptical shaped (Saeed and Salam 2013).

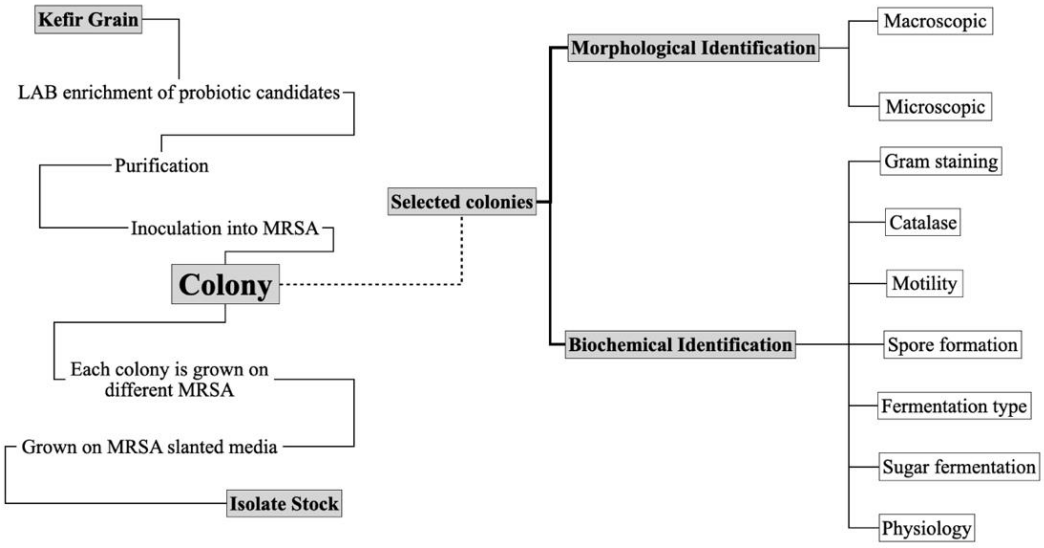


**Figure 1.** Isolation and identification methods in kefir grain

**Phenotypic identification with a basis of biochemistry**

All isolates showed Gram-positive, negative catalase, non-motile, non-spore, not producing $CO_2$, mostly homofermentative (n=15) and only one heterofermentative (n=1) (Table 2). Accordingly, the isolates were closer to those of LAB, which, according to (Zoumpopoulou et al. 2018), have Gram-positive that is characterized by peptidoglycan walls made of peptide and carbohydrate. Gram-negative characteristics are not the characteristics of LAB, so when the test observed any Gram-negative, no further test was needed to pursue. In this study, all 16 isolates were Gram-positive, so they were tested for further parameters. LAB does not produce catalase enzymes that can degrade hydrogen peroxide and oxygen, as indicated by no gas bubbles formed during the catalase test (Ibrahim et al. 2015). Non-motile is another LAB characteristic, as shown by non-existent patches that grow around the loop puncture (Susilawati 2016). In addition, LAB does not produce spores (Laily et al. 2013). The heterofermentative characteristics of LAB consist of *Oenococcus*, *Leuconostoc*, and *Weisella* strains, while some of *Lactobacillus* and *Pediococcus* are either homofermentative or heterofermentative (Axelsson 2004). Based on this characterization, all 16 isolates have shown the expected characteristics of LAB.

All isolates in this study that could ferment carbohydrates were glucose, D-galactose, glycerol, D-fructose, D-sorbitol, D-lactose, and D-saccharose (Table 3). Fermentation is the only way LAB produces energy. The fermentation activity of each carbohydrate isolate differs from one species to another (Jay et al. 2008). Gunkova et al. (2021) have proven that *Streptococcus thermophilus* and *Lactobacillus delbrueckii* spp show the slowest growth when fermented in the maltose media, while *Leu. Mesemteroides* can rapidly ferment sucrose. Maltose, sucrose, and lactose can be metabolized fast by *L. lactis* spp. *lactis, L. lactis* ssp. *cremoris* and *L. lactis* spp. *lactis biovar*. Several types of LAB show different activities of carbohydrate fermentation. Lactose is a vital source of carbohydrates for LAB growth and is found in fermented milk products. Also, LAB can ferment sucrose, maltose, and galactose (Gunkova et al. 2021).

**Table 1**. Macroscopic Identification of Isolates

| Isolate Code | Macroscopic | | | | | Microscopic | |
|---|---|---|---|---|---|---|---|
| | Color | Shape | Edge | Surface | Elevation | Cell shape | Cell structure |
| PD | Milky white | Circular | Intact | Smooth and shiny | Convex | Coccus | Mono |
| DW | Milky white | Circular | Intact | Smooth and shiny | Convex | Bacill | Mono |
| BP | Milky white | Circular | Intact | Smooth and shiny | Convex | Bacill | Mono |
| MQ | Milky white | Circular | Intact | Smooth and shiny | Convex | Coccus | Mono |
| QF | Milky white | Circular | Intact | Smooth and shiny | Convex | Bacill | Mono |
| JS | Milky white | Circular | Intact | Smooth and shiny | Convex | Bacill | Mono |
| DS | Milky white | Circular | Intact | Smooth and shiny | Convex | Coccus | Mono |
| ES | Milky white | Circular | Intact | Smooth and shiny | Convex | Bacill | Mono |
| SS | Milky white | Circular | Intact | Smooth and shiny | Convex | Bacill | Mono |
| DI | Milky white | Circular | Intact | Smooth and shiny | Convex | Coccus | Mono |
| DH | Milky white | Circular | Intact | Smooth and shiny | Convex | Coccus | Mono |
| AN | Milky white | Circular | Intact | Smooth and shiny | Convex | Coccus | Mono |
| RJ | Milky white | Circular | Intact | Smooth and shiny | Convex | Coccus | Mono |
| NR | Milky white | Circular | Intact | Smooth and shiny | Convex | Coccus | Mono |
| AS | Milky white | Circular | Intact | Smooth and shiny | Convex | Bacill | Mono |
| SM | Milky white | Circular | Intact | Smooth and shiny | Convex | Coccus | Mono |

**Table 2**. Biochemical Test of Lactic Acid Bacteria

| Isolate code | Cat Gram | Catalase | Motility | Spore | Type of fermentation | $CO_2$ production |
|---|---|---|---|---|---|---|
| PD | Positive (+) | - | Non-motile | - | Homofermentative | - |
| DW | Positive (+) | - | Non-motile | - | Homofermentative | - |
| BP | Positive (+) | - | Non-motile | - | Homofermentative | - |
| MQ | Positive (+) | - | Non-motile | - | Homofermentative | - |
| QF | Positive (+) | - | Non-motile | - | Homofermentative | - |
| JS | Positive (+) | - | Non-motile | - | Homofermentative | - |
| DS | Positive (+) | - | Non-motile | - | Homofermentative | - |
| ES | Positive (+) | - | Non-motile | - | Heterofermentative | - |
| SS | Positive (+) | - | Non-motile | - | Homofermentative | - |
| DI | Positive (+) | - | Non-motile | - | Homofermentative | - |
| DH | Positive (+) | - | Non-motile | - | Homofermentative | - |
| AN | Positive (+) | - | Non-motile | - | Homofermentative | - |
| RJ | Positive (+) | - | Non-motile | - | Homofermentative | - |
| NR | Positive (+) | - | Non-motile | - | Homofermentative | - |
| AS | Positive (+) | - | Non-motile | - | Homofermentative | - |
| SM | Positive (+) | - | Non-motile | - | Homofermentative | - |

**Table 3**. Sugar fermented test by lactic acid bacteria isolates

| Isolate code | Glucose | D-galactose | Glycerol | D-fructose | D-sorbitol | D-lactose | D-saccharose |
|---|---|---|---|---|---|---|---|
| PD | + | + | + | + | + | + | + |
| DW | + | + | + | + | + | + | + |
| BP | + | + | + | + | + | + | + |
| MQ | + | + | + | + | + | + | + |
| QF | + | + | + | + | + | + | + |
| JS | + | + | + | + | + | + | + |
| DS | + | + | + | + | + | + | + |
| ES | + | + | + | + | + | + | + |
| SS | + | + | + | + | + | + | + |
| DI | + | + | + | + | + | + | + |
| DH | + | + | + | + | + | + | + |
| AN | + | + | + | + | + | + | + |
| RJ | + | + | + | + | + | + | + |
| NR | + | + | + | + | + | + | + |
| AS | + | + | + | + | + | + | + |
| SM | + | + | + | + | + | + | + |

**Table 4**. Physiology of lactic acid bacteria isolates against the changes in temperature, pH, and salinity

| Isolate code | Temperature (°C) | | | pH | | Salinity level | |
|---|---|---|---|---|---|---|---|
| | 10 | 37 | 45 | 4.4 | 4.6 | 6.5 | 18 |
| PD | - | + | + | + | - | + | - |
| DW | - | + | + | + | - | + | - |
| BP | - | + | + | + | - | + | - |
| MQ | - | + | + | + | - | + | - |
| QF | - | + | + | + | - | + | - |
| JS | - | + | + | + | - | + | - |
| DS | - | + | + | + | - | + | - |
| ES | - | + | + | + | - | + | - |
| SS | - | + | + | + | - | + | - |
| DI | - | + | + | + | - | + | - |
| DH | - | + | + | + | - | + | - |
| AN | - | + | + | + | - | + | - |
| RJ | - | + | + | + | - | + | - |
| NR | - | + | + | + | - | + | - |
| AS | - | + | + | + | - | + | - |
| SM | - | + | + | + | - | + | - |

The characteristics of LAB isolates can be analyzed based on their physiological properties, including their ability to grow at different temperatures, pH, and salt concentrations (see Table 4). The basis of LAB classification was determined from LAB capacity to grow at 10, 37, and 45°C. The results showed that the isolates grew at 37 and 45°C, but the former was the best temperature to grow for the majority of LAB. LAB can live in the range of 10-45°C (Laily et al. 2013), and yet, some species (e.g., *Lactobacillus* and *Pediococcus)* cannot live or grow in this range, while others (e.g., *Lactococcus* and *Leuconostoc*) grow well at 10°C but not at 45°C (Axelsson 2004).

Different classifications of LAB are based on the pH (4.4 and 9.6), which indicated that all isolates could grow in an environment at pH 4.4, but no growth whatsoever was detected at pH 9.6. *Pedioccoccus* showed the ability to grow at acidic pH; at alkaline pH was *Aerococcus* and *Tetragenococcus*, and at both pH conditions was *Enterococcus.* Meanwhile, depending on the species, some *Lactococcus, Lactobacillus, Vagococcus, Leuconostoc, Oenococcus,* and *Weisella* can grow at acidic pH while others cannot (Axelsson 2004).

The capacity to grow at different salt concentrations (6.5 and 18%) was tested on all isolates. The results showed that all isolates could grow at 6.5% salt concentration but not 18%. It indicates that low salt concentration (6.5%) is ideal for optimum growth of bacteria. *Aerococcus, Enterococcus, Tetragenococcus,* and *Weisella* could grow at 6.5% salt concentration, while *Tetragenococcus* was at 18%. Some *Lactobacillus, Leuconostoc, Oenococcus*, and *Pediococcus* could grow at 18%, while some could not at 6.5%, depending on the species (Axelsson 2004).

## Discussion

The criteria for identifying LAB proposed by Orla-Jensen in early 1919 were based on morphology, glucose fermentation method, range of temperature, and glucose utilization pathways (Quinto et al. 2014; Vinderola et al. 2019). All isolates obtained in this study were strongly assumed to be included in the LAB group. LAB is a microbe with gram-positive, non-motile properties, cocci and bacilli, and ferment carbohydrates mainly into lactic acid (Reis et al. 2012). All bacterial isolates are divided into nine groups. The isolation carried out on Tibetan kefir grain based on its phenotypic characteristics produces isolates with properties that resemble *Lactococcus, Lactobacillus,* and *Leuconostoc* (Gao et al. 2012). The *Lactobacillus* genus is of Gram positive, non-motile, and non-sporulation. However, while *Lactobacillus* is a facultative anaerobic bacteria that is tolerant to acidic conditions, it can be either homofermentative or heterofermentative (Alkema et al. 2016)

Compared to the phenotypic characteristics using the Bergey manual on systematic bacteriology (Oren and Garrity 2014), this study showed some resemblance. Isolates coded with PD, MQ, DS, DI, DH, AN, RJ, NR, and SM are the colony groups with milky white color, smooth surface, Gram-positive, negative catalase, and cocci cells. Furthermore, physiological and biochemical tests showed that these groups belonged to the *Lactococcus* category. Isolates DW, BP, QF, JS, ES, SS, and AS have a smooth surface, Gram positive, negative catalase, bacillus-shaped along with physiological and biochemical properties that show characteristics as *Lactobacillus* strains. Based on Bergey's guidebook, *Lactobacillus* has transparent color characteristics, but Bhardwaj et al. (2012) showed that *Lactobacillus* can also be white, cream, greenish white, grayish white, and creamish white. Therefore, DW, BP, QF, JS, ES, SS, and AS isolates in this study can be grouped into *Lactobacillus* strains. Moreover, the results of physiological analysis on growth ability at different pH showed that isolates could grow at 4.4. Therefore, the isolates observed in this study resembled *Lactococcus* and *Lactobacillus.*

The identification method using phenotypic characteristics has multiple benefits, including the use of simple tools for analysis. However, in some major cases, identifying species through phenotypic characteristics is inaccurate because many different LAB species have similar phenotypic characteristics (Merilä and Hendry 2014). Microorganisms can be identified up to species level using genotypic identification. Meanwhile, the DNA-based genotypic method (molecular biology) offered a better solution for identifying LAB. Combining phenotypic and genotypic identification is preferred because it produces more optimum results (Ritchie et al. 2015). Accordingly, further study is needed in genotypic identification to strengthen the results of the present study up to the species level.

In conclusion, LAB have been isolated from kefir grain and subjected to phenotypic and biochemical identification. Sixteen isolates identified from a scientific approach are derived from several strains, including 9 *Lactococcus* isolates and 7 *Lactobacillus* isolates. It takes genotypic identification to promote the identification process up to the species level.

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