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ISOLATION AND IDENTIFICATION OF GOAT MILK-DERIVED *Lactobacillus paracasei* M104 AND *Pediococcus pentosaceus* M103 AND THEIR POTENTIAL USE AS STARTER CULTURE FOR FERMENTATION

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ABSTRACT

The aims of this study were to isolate and identify lactic acid bacteria from the fresh milk of crossbred Peranakan Etawah goats in Yogyakarta, Indonesia and assess their potential utility in dairy fermentation. Fresh milk samples were collected from three different farms and plated into de Man Rogosa and Sharpe (MRS) agar supplemented with 0.5% ox bile. Colonies were purified with a streaking method followed by morphological and biochemical analysis using Gram staining, a catalase test, tests of motility and spore formation and growth at different temperatures. Molecular identification was based on nucleotide sequencing of 16S rRNA genes. Four isolates, M101, M102, M103 and M104, were identified. Certain features of isolates M101 and M102 were homologous with *Lactococcus garvieae*, isolates M103 and M104 showed a degree of homology with *Pediococcus pentosaceus* and *Lactobacillus paracasei* subsp. *paracasei* respectively. Selected isolates were used to ferment milk at 37°C for 10 hours. After 10 h, milk fermented with *Lactobacillus paracasei* M104 had a pH of 4.21±0.07 and acidity of 1.13±0.05. Milk fermented with *Pediococcus pentosaceus* M103 had a pH of 4.34±0.03 with acidity of 1.18±0.05. *Lactococcus garvieae* had limited ability to acidify milk, producing only a slight change in pH over 10 h. There was no significant difference (P>0.05) in viscosity between milk fermented with *Lactobacillus paracasei* M104 and milk fermented with *Pediococcus pentosaceus* M103. Total viable cells were similar between milk fermented with *Lactobacillus paracasei* M104 and milk fermented with *Pediococcus pentosaceus*. On the basis of their capacity to acidify milk, *Pediococcus pentosaceus* M103 and *Lactobacillus paracasei* M104 were selected for further investigation.

Keywords: Goat milk, lactic acid bacteria, molecular identification, fermentation

INTRODUCTION

Goat farming is an attractive enterprise for small-scale farmers in developing countries and those working sub-prime agricultural land (Pirisi *et al.*, 2007) as goats are well-adapted to grazing on poor or marginal land. The capacity for both meat and milk production is another benefit. The main use of goat milk is for cheese-making in small local dairies, particularly in Mediterranean and south-east European countries, although there are also some big cheese factories in Western Europe (Pirisi *et al*., 2007). Goat milk can be used as a substitute for cow milk in cases of allergy to bovine milk (Kongo *et al*.,1996). To date goat milk has been fermented using lactic acid bacteria (LAB) as a starter culture (del Campo *et al*., 2005). In Indonesia, goat milk has been used for cheese production based on LAB as starters obtained from commercial sources. A number of LAB species have been used for milk fermentation, for example *Lactococcus lactis* in cheese production and *Lactobacillus casei* for souring milk. LAB species used in Indonesian dairy productions are usually isolated from gastrointestinal tract (GIT) or from food products; there have been no reports of use of LAB isolated from local goat milk.

LAB are frequently associated with food and feed fermentation (Axelsson, 2004). LAB species are indigenous to food habitats such as plant-derived products and milk environments. LAB are also naturally associated with the animal mucosae, including the mucosae of the small intestine, colon and vagina. The same LAB species are frequently isolated from diverse sources, implying wide distribution and adaptation to a range of environments (Makarova *et al*., 2006). During fermentation LAB produce organic acids, mainly lactic acid and acetic acid, that decrease pH and increase acidity thus causing rapid acidification of fresh milk. This produces fermented milk products with low pH, such as yoghurt, sour milk and other products. Fermenting milk increases the availability of its nutrients. The aim of this study was to isolate and identify LAB from the fresh milk of crossbred Peranakan Etawah (PE) goats, and evaluate their potential for use as starter culture in milk fermentation.

MATERIALS AND METHODS

Fresh milk preparation, bacterial isolation and identification

Fresh goat milk samples were obtained from three different farms in Yogyakarta, Indonesia. Fresh milk was cooled immediately after milking in an ice box and transported to the laboratory for analysis. An 1ml aliquot of fresh goat milk was added to 9 ml of 0.1% (w/v) sterile peptone water to obtain 10^{-1} dilution. After three-fold serial dilution 0.1 ml aliquots were surface plated on de Man Rogosa and Sharpe (MRS) agar (Merck) supplemented with 0.15% (w/v) ox bile then incubated anaerobically at 37°C for 48 hours. White colonies visible on the plate after incubation were subjected to morphological and physiological analysis, including Gram staining, a catalase test, assessments of shape, spore formation, motility and CO_2 and NH_3 production and comparison of growth at 10ºC and 45ºC. These screening tests were used to select colonies with LAB characteristics which were then subjected to molecular identification using 16S rRNA gene amplification.

Amplification of 16S rRNA gene

Amplification of a 518bp fragment of16S rRNA gene was carried out using PCR with primers based on the conserved region of 16S rRNA gene, namely a forward primer *plb16* (5-AGAGTTTGATCCTGGCTCAG-3) and a reverse primer *mlb16* (5-GGCTGCTGGCACGTAGTTAG-3) (Martin *et al*., 2009). Gene amplification was performed using a PCR thermal cycler. The PCR protocol was as follows: denaturation at 95°C for 30s, annealing at 56°C for 30s, elongation at 72°C for 45s (30 cycles) and a final extension at 72°C for 10 min. Amplified bands were resolved by electrophoresis in 1.6% (w/v) agarose gels and visualised using ethidium bromide staining.

DNA sequencing and phylogenetic analysis

The amplified DNA was sequenced using an Applied Biosystem 3730-XL Analyser at 1st Base Sequencing, Kuala Lumpur, Malaysia. The resulting sequences were used to search the National Centre for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) algorithm; the isolates were identified on the basis of the sequences which produced the best match (>97%). Molecular Evolutionary Genetics Analysis (MEGA) 6.0 was used to construct a phylogenetic tree of the sequences based on the neighbour-joining algorithm (Saitou & Nei, 1987).

Milk fermentation

Fresh milk was pasteurised at 85°C for 30 min, followed by cooling to 37°C. Pasteurised milk was inoculated with bacterial cultures (2% v/v) and thoroughly stirred. The inoculated milk was incubated at 37°C for 10h; pH and acidity were analysed every 2 hours during the fermentation period. The pH value of the fermented milk was measured with a pH meter (Hanna model, Romania). Titratable acidity was measured as percentage lactic acid by titrating 0.1 N of NaOH using phenolphthalein as indicator (Hadiwiyoto, 1994). The titration method (AOAC Official Method, 1995) was used for lactose analysis.

Apparent viscosity and syneresis

The apparent viscosity of samples was analysed at 29°C using the procedure described by Tuncturk (2009) using a Brookfield digital rheometer model DV III (Brookfield Engineering Laboratories Inc., Massachusetts, USA) with spindle numbers 62 and 63, spindle speed 60 rpm. Syneresis was measured using centrifugation (Keogh and O'Kennedy, 1998). A 15 g aliquot of sample was centrifuged at 1500 rpm for 20 min at 4°C. The supernatant was collected and weighed and the extent of syneresis was calculated using following equation:

$$\text{Syneresis (\%)} = \frac{\text{Weight of supernatant (g)}}{\text{Weight of sample (g)}} \times 100\%$$

Total viable cells

An 1 ml aliquot of fermented milk was collected from all samples. Aliquots were diluted seven-fold in a sterile solution of 0.85% (w/v) NaCl, then plated onto MRS agar and incubated at 37°C for 24 to 48 h. The colonies were then counted and total bacterial numbers per sample were estimated.

Lactic acid concentration using High Performance Liquid Chromatography (HPLC)

Lactic acid measured using HPLC was carried out according to the procedure described by Bevilacqua and Califano (1989) using a reverse-phase C8 column at room temperature, a mobile phase of 0.5% w/v buffer ($(NH_4)_2HPO_4$ at pH 2.24 with H_3PO_4)-0.4% (v/v) acetonitrile and 1.2 mL/min flow rate with UV detection at 214 nm.

Data analysis

Data on pH, acidity, lactose content, lactic acid concentration, viscosity and syneresis were analysed statistically using paired *T*-tests with statistical significance accepted at $P<0.05$.

RESULTS AND DISCUSSION

Bacterial Identification and Selection

We obtained 4 isolates with the morphological and physiological characteristics of LAB (Table 1). LAB are usually characterised as Gram-positive, aerobic or facultative anaerobic, non-motile asporogenous rods and cocci with the ability to ferment carbohydrate with lactic acid as the main fermentation product (Franz *et al*., 2010). Preliminary identification was based on morphological and physiological analysis, ability to produce CO_2, growth at different temperatures (10°C, 37°C and 45°C), pHs (4.4 and 9.6) and in media containing 6.5% NaCl. The characteristics of all isolates are presented in Table 1.

Table 1 Morphological and biochemical identification of isolates

Isolates	Form	CO_2	Growth temperature			NaCl 6.5%	Growth pH		Genus*
			10°C	37°C	45°C		4.4	9.6	
M101	Cocci	-	-	+	+	-	+	-	*Pediococcus*
M102	Cocci	-	-	+	+	+	-	-	*Lactococcus*
M103	Cocci	-	-	+	+	-	+	-	*Pediococcus*
M104	Bacilli	-	-	+	+	+	+	-	*Lactobacillus*

* Based on Axelsson's (2002) classification of LAB.

Comparison of the fermentation patterns of all isolates with Axelsson's (2002) classification of LAB resulted in identification of three genera of LAB; M101 and M103 were identified as *Pediococci*, M102 as *Lactococcus* and M104 as *Lactobacillus* (Table 1). All isolates were homofermentative without CO_2 production and isolates M101 and M103 were intolerant of salt (NaCl 6.5%). 16S rRNA gene sequencing suggested that isolates M101 and M102 were close homologues (97% similarity) of *Lactococcus garvieae* strain 29; isolate M103 was a close homologue (96% similarity) of *Pediococcus pentosaceus* strain LAB6 and isolate M104 was a close homologue (97% similarity) of *Lactobacillus paracasei* subsp. *paracasei* strain X212 (Figure 1).

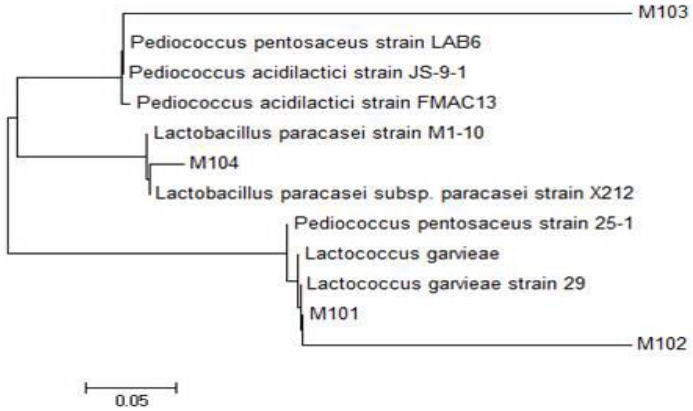

Figure 1 Phylogenetic tree showing the genetic relationship of selected isolates to other LAB

This study identified *L. garvieae*, *P. pentosaceus* and *L. paracasei* in fresh milk from crossbred Peranakan Etawah goats in Indonesia. *L. garvieae* is often associated with fermented dairy products; Morea, Baruzzi and Cocconcelli (1999) have previously reported its presence in goat milk. *L. garvieae* is one of main bacterial populations in traditional production of mozzarella-type cheese from goat milk and forms part of the autochthonous bacterial population in various Italian and Greek cheeses (Fortina *et al*., 2007). *L. garvieae* has also been reported to occur in the milk of Kabyle goats from Algeria (Badis *et al.*, 2004). This study reports the presence of *L. garvieae* in fresh milk from crossbred Peranakan Etawah goats.

P. pentosaceus is part of the natural microflora of vegetables and dairy products (Carraro *et al*., 2011) and has also been found in human breast milk (Osmanagaogl *et al*., 2013). *P. Pentosaceus* appears to be a good candidate as starter culture for dairy fermentation as it grows well at 45°C, is homofermentative and has a long history as being safe for consumption. In other reports, *P. pentosaceus* isolated from traditional dairy products was good at curdling milk.

L. paracasei has previously been isolated from goat milk (Badis *et al*., 2004). This species has been used as starter culture for probiotic fermented milk (Kristo *et al*., 2003; Patrignani *et al*., 2009). It inhibits the activity of yeasts on the surface of dairy products such as yoghurt or cheese at refrigerator temperatures (6°C) without an influencing their quality (Schwenninger and Meile, 2004).

Acidification Capability

The acidifying capacity of the selected isolates was evaluated by using them to inoculate individual samples of sterile skimmed milk (18% w/v) and incubating at 37°C for 10 h. *P. pentosaceus* M103 and *L. paracasei* M104 exhibited fast acidification activity (Figures 2 and 3), whereas *L. garvieae* M101 and M102 exhibited slow acidification activity (data not shown). The pH and acidity of milk samples inoculated with *L. paracasei* M104 and *P. pentosaceus* M103 during fermentation are presented in Figures 2 and 3, respectively. At the final pH measurement, isolates *L. garvieae* M101 and M102 had pHs of 5.18 ± 0.12 and 5.24 ± 0.17 respectively, whilst fermentation using *P. pentosaceus* M103 and *L. paracasei* M104 resulted in samples with pHs of 4.34 ± 0.03 and 4.21 ± 0.07, respectively (Figure 2).

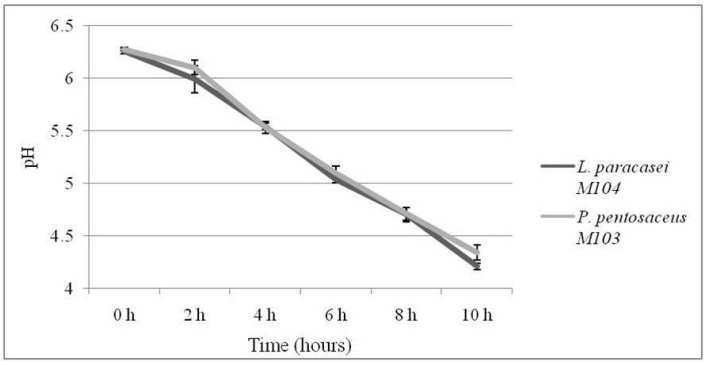

Figure 2 Changes in pH during incubation with different starter cultures

Figure 2 showed that during fermentation pH decreased gradually whilst acidity increased significantly ($P<0.05$) (Figure 3). The acidity of milk fermented with *L. paracasei* M104 increased from 0.31% ± 0.01 to 1.13 ± 0.05 and the acidity of milk fermented with *P. pentosaceus* M103 increased from 0.31% ± 0.01 to 1.18 ± 0.05 (Figure 3); milk fermented with *L. paracasei* M104 or *P. pentosaceus* M103 had similar acidity and pH values.

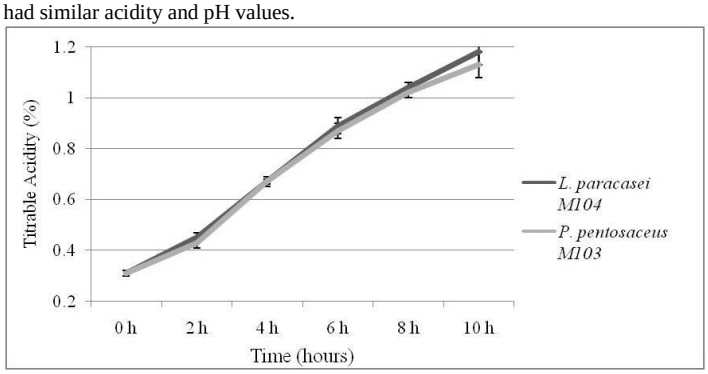

Figure 3 Changes in acidity during incubation with different starter cultures

According to Robinson (2002), acidification is the main indicator of the progress of fermentation. LAB produce organic acids from lactose enzymatically, resulting in formation of curds with distinctive flavours. In this study lactose content and lactic acid concentration were measured before and after fermentation (Table 2). During fermentation the lactose content of milk decreased from 4.6%±0.39 to 3.15%±0.35 when fermented with *L. paracasei* M104, and from 4.6% ± 0.39 to 3.2% ± 0.22 when fermented with *P. pentosaceus* M103 (Table 2), whilst the lactic acid concentration increased from 0.1422% ± 0.021 to 0.1905% ± 0.0145 and from 0.1422% ± 0.021 to 0.1768% ± 0.034 during fermentation with *L. Paracasei* M104 and *P. pentosaceus* M103 respectively (Table 2).

Table 2 Changes in lactose content (%) and lactic acid (%) after 10h fermentation

Starter Culture	Time (hour)	Lactose (%)	Lactic Acid (%)
L. paracasei M104	0	4.60 ± 0.39[a]	0.1422 ± 0.0210[b]
	10	3.15 ± 0.35[b]	0.1905 ± 0.0145[a]
P. pentosaceus M103	0	4.60 ± 0.39[a]	0.1422 ± 0.0210[b]
	10	3.24 ± 0.22[b]	0.1768 ± 0.034[a]

Values expressed as mean ± standard deviation of three replicates
Means within each column with different superscript letters indicate statistically significant differences (P<0.05)

The increase in acidity during fermentation resulted in coagulation of proteins and curd formation, leading to increased viscosity and syneresis. We therefore measured apparent viscosity and syneresis after fermentation; these data are presented in Table 3. Before fermentation milk had a viscosity of 1.82cP ± 0.12 (Table 3). Milk fermented with *P. pentosaceus* M103 had significantly ($P<0.05$) higher apparent viscosity than milk fermented with *L. paracasei* M104 (1475cP ± 15 and 1075cP ± 25 respectively, at the end of the fermentation period) (Table 3). There were no significant differences (P>0.05) in syneresis between milk fermented with *L. paracasei* M104 or *P. pentosaceus* M103 (84.94% ± 1.6 and 84.94% ± 2.2 respectively) (Table 3).

Table 3 Viscosity (cP) and syneresis (%) before and after fermentation

Starter culture	Viscosity		Syneresis
	0 h	10 h	(10 h)
L. paracasei M104	1.82±0.12[a]	1075±25[b]	84.94±1.6
P. pentosaceus M103	1.82±0.12[a]	1475±15[c]	84.94±2.2

Values expressed as mean ± standard deviation of three replicates
Means within each column with different superscript letters indicate statistically significant differences (P<0.05)

Starter cultures can be defined as a microbial biomass, consisting of a large number of cells in the logarithmic phase of growth, which can be added to raw food products to accelerate fermentation processes (Leroy and De Vuyst, 2004). *P. pentosaceus* M103 and *L. paracasei* M104 (Figures 2 and 3) showed fast acidification activity, whereas *L. garvieae* M101 and M102 showed slow acidification activity (data not shown). The primary function of LAB in industrial dairy fermentation is use of lactose (de Vos and Vaughan, 1994). Lactic acid accumulation decreases pH and improves the acidity of products. Fermented dairy products usually have a pH of 4.6 or lower (Chandan, 2006). In this study products fermented with *L. garvieae* M101 and M102 had pHs of no 4.34 ± 0.03 and 4.21 ± 0.07, respectively (Figure 2), i.e. within the required range. A previous study (Widodo *et al.*, 2014) reported that pH 4.5 was achieved after 5 hours of goat milk fermentation using mono-species starter cultures of *Lactobacillus acidophilus* FNCC-0029 or *Lactobacillus casei* FNCC-0051. In this study a pH of 4.5 was obtained after 9 hours of fermentation with *P. pentosaceus* M103 or *L. paracasei* M104 as starter, indicating that fermentation with these species was slower than that of *Lactobacillus acidophilus* FNCC-0029 or *Lactobacillus casei* FNCC-0051. The final acidities of milk fermented with *L. paracasei* M104 or *P. pentosaceus* M103 were 1.13% ± 0.05 and 1.18% ± 0.05 respectively (Figure 3), within the range of the Indonesian national standard (SNI), 0.5 to 2.0% (Badan Standarisasi Nasional, 1998).

Higher acidity stimulates syneresis, thereby improving the viscosity of fermented dairy products (Tamime and Robinson, 1999). In this study use of *P. pentosaceus* M103 or *L. paracasei* M104 for milk fermentation resulted in products with the same final acidity (Table 2), but different viscosities (Table 3). Keogh and O'Kennedy (1998) suggested that differences in viscosity are probably due to variations in protein ratios or ion values, and may also be affected by incubation and storage conditions.

Total Viable Cells

Data on the numbers of viable *L. paracasei* M104 and *P. pentosaceus* M103 before and after fermentation are presented in Table 4. At the end of fermentation there was no significant difference (P>0.05) in the number of viable cells in samples fermented with *P. Pentosaceus* M103 or *L. Paracasei* M104 (Table 4). After fermentation the total number of viable *L. paracasei* M104 cells was 9.23 $\log_{10}$ CFU/ml, an increase of 1.4 $\log_{10}$ CFU/ml, whereas the total number of viable *P. pentosaceus* M13 cells was 9.63 $\log_{10}$ CFU/ml, an increase of 1.9 $\log_{10}$ CFU/ml (Table 4).

Table 4 Viable cells ($\log_{10}$cfu/ml) in milk before and after fermentation

Starter culture	Viable count (CFU/ml)	
(2%, w/v)	0 h	10 h
L. paracasei M104	7.817±0.053[a]	9.238±0.330[b]
P. pentosaceus M13	7.719±0.159[a]	9.637±0.510[b]

Values expressed as mean ± standard deviation of three replicates
Means within each column with different superscript letters indicate statistically significant differences (P<0.05)

The total numbers of viable *P. pentosaceus* M103 and *L. paracasei* M104 after fermentation were 9.637 ± 0.510 and 9.238 ± 0.330 $\log_{10}$ CFU/ml respectively; the minimum value required for functional activity in fermented milk products is 10^6 CFU/ml (Shah, 2000). We did not evaluate the probiotic capability of *P. pentosaceus* M103 or *L. paracasei* M104; however several other studies have classified both species as probiotic (Kristo *et al*., 2003; Osmanagaoglu *et al*., 2013; Patrignani *et al*., 2009).

CONCLUSION

This study identified four species of LAB in the milk of Peranakan Etawah goats. Isolates identified as *Lactobacillus paracasei* M104 and *Pediococcus pentosaceus* M103 were shown to acidify skimmed milk effectively and had potential uses as starter culture for milk fermentation.

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