

Isolation of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* as Starter Culture Candidate Originated from Indonesian Cow's Milk

Danish Andrian[†], Denny Rizkinata[†], Tan Steven Ryan Susanto, Jap Lucy*, and Tan Tjie Jan

Department of Biology, Universitas Pelita Harapan, Tangerang 15811, Indonesia

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Streptococcus thermophilus, *Lactobacillus delbrueckii*, *Lactobacillus fermentum* and *Lactobacillus casei* were successfully isolated from indigenous Indonesian fresh milk based on the general morphological and biochemical classification as described in Bergey's manual. Verification was conducted by sequencing of 16S rRNA after selection using the classification method mentioned in the manual. All isolates exhibited antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* in the well diffusion test. The susceptibilities of the isolated *S. thermophilus* 24/S1 and *L. delbrueckii* 94/L4 against 22 different antibiotics were determined by the disc diffusion method and variable susceptibility patterns were observed. Both isolates were susceptible to amoxicillin, the most commonly prescribed antibiotic, and resistant to sulfonamide. The presence of a plasmid was not detected after extraction. *S. thermophilus* 24/S1 and *L. delbrueckii* 94/L4 starter cultures were prepared for yogurt production after 9.5 h of incubation and the yogurt was evaluated for its flavor and quality by 30 volunteers. A score of 4.93 ± 0.45 out of 7 was obtained as compared to the yogurt prepared using commercial starter cultures which yielded a score of 4.76 ± 0.30 out of 7.

Keywords: Bergey's manual, starter culture, fresh milk, *Lactobacillus delbrueckii*, *Streptococcus thermophilus*, yogurt

Introduction

The discoveries on the advantages of probiotic have made an outstanding breakthrough in food as well as healthcare sectors. A particular species or assortments of harmless microbes have been expansively studied demonstrating favourable results that worth extensive assessments in order to combat common infections i.e. *Salmonella* sp. [1, 2], Enterohemorrhagic *Escherichia coli* [3], *Streptococcus pneumoniae* [4], *Klebsiella pneumoniae* [5] and even the obscure *Plasmodium* spp. [6] infections. Probiotic is widely applied to human as well as animals. Current studies relating to probiotic roles in

improving gut health [7], addressing antibiotic resistance problem [8], ameliorating allergies conditions [9] as well as improving livestock's wellbeing and productions [10] have been thoroughly described. These beneficial microorganisms were isolated from various sources i.e. traditional fermented products [11], gastrointestinal tract [12], faeces [13], soil [14] and dairy products [15]. The genus of probiotic bacteria ranged from: *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Streptococcus*, *Pediococcus*, *Leuconostoc*, *Bacillus* and *Escherichia*. Each species in the probiotic genera confers variable immunomodulatory effects in health or in the course of infections [16, 17].

Milk is an exceptionally nutritive dairy product that provides an ideal niche for lactic acid bacteria (LAB) such as *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Streptococcus* and *Enterococcus* to thrive in. The milk microbiota of an animal host delineates health status of the

*Corresponding author

Tel: +62-21-5470901, Fax: +62-21-5460910

E-mail: jap.lucy@uph.edu

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[†]These authors contributed equally to this work.

organism [18]. Although recognition on the benefit of probiotic has grown in the last few decades, which led to the rapid expansion on the world of probiotic, the *Lactobacillus* genera is still one of the most widely known and applied probiotic [16]. *S. thermophilus*, a thermophile growing at 45°C, on the other hand was involved in the making of fermented dairy product especially yogurt and cheese [19]. It is the only known species of the genus of *Streptococcus* found in starter cultures [20]. *S. thermophilus* is widely used along with *L. delbrueckii* as a starter culture in yogurt manufacture. *S. thermophilus* thrives in and ferments milk to produce yogurt by slightly increasing acidity and depleting oxygen of the milk which is conducive for *L. delbrueckii* to grow rapidly in the medium and forms valine. Valine formation is necessary for *S. thermophilus* to multiply [20]. Furthermore, *S. thermophilus* is known to produce beneficial biometabolite [21] and also making yogurt friendly to lactose intolerant sufferers [22]. The isolation of *L. delbrueckii* and *S. thermophilus* were conducted to assess the availability of the genera and species from local dairy products, particularly from Indonesia indigenous fresh cow milk, which is functional as a starter culture in yogurt production.

Starter cultures are of great industrial significance on the efficient manufacturing, flavor, and texture development of fermented dairy foods. The public demands on fermented dairy products in Indonesia are absolutely increasing [23]. The growing awareness on the beneficial effect of probiotic consumption has resulted on the rapid advancement in the development of starter culture throughout the world. Commercial starter culture has been widely established in many developed country [24]. The release of local starter culture utilized in dairy products fermentation process to the market will promote awareness on the benefits of these products and support the development of local market. We aim to isolate starter culture of *S. thermophilus* and *L. delbrueckii* isolated from indigenous milk originated from Indonesia to be made into dairy fermented product such as yogurt.

Materials and Methods

Isolation of *Lactobacillus delbrueckii* and *Streptococcus thermophilus*

Fresh milk sample was obtained from Holstein Frie-

sian (HF) cattle from Djampang Farm, Bogor, West Java, Indonesia. Samples were collected aseptically into sterile collecting tubes, stored in an ice-box and transported to the laboratory for analysis. Samples were serially diluted with 0.85% (wt/vol) sterile saline supplemented with 0.1% (wt/vol) peptone as nutrients source to maintain viability of the isolates in the sample. The dilutions (10^{-1} – 10^{-3}) were spread plated on de Man, Rogosa, and Sharpe agar (MRSA; Merck, Germany) and were incubated microaerobically for 24 h at 37°C. A total of 204 isolates were transferred and re-cultured on sterile MRSA plate for purification. Isolates were morphologically and biochemically characterized through manual as described by Bergey's Manual of Systemic Bacteriology for selection of potential *L. delbrueckii* and *S. thermophilus* species [25].

First, general characterization of isolates by catalase, Gram-staining, endospore and acid-fast staining to match characteristics of the genus *Lactobacillus* (catalase-negative, Gram-positive, rod shaped, non-endospore forming and non-acid-fast bacteria), and of *S. thermophilus* (catalase-negative, Gram-positive, cocci shaped, non-endospore forming and non-acid-fast bacteria) was conducted. Selected isolates were further analyzed for their ability to grow on MRS broth (MRSB; Merck, Germany) incubated at selected temperatures (10°C and 45°C) and also incubated under selected salinity concentration (2%, 4% and 5%). Growth was measured through spectrophotometry reading at wavelength 600 nm (OD_{600}). Each of the colonies' morphology (shape, margin, elevation and color) and their ability to ferment variety of sugars (mono-saccharides: glucose, fructose; di-saccharides: lactose, maltose, sucrose; tri-saccharide: raffinose; and sugar-alcohol: mannitol and sorbitol) were also analyzed to categorize and select representatives for 16S rRNA sequencing [25].

16S rRNA sequencing

A representative from each group of isolates displaying similar patterns was selected for molecular identification. Selected isolates were cultured overnight and bacterial genomic DNA extraction was conducted using Wizard[®] Genomic DNA Purification Kit (Promega, USA) and the extracted DNA was quantified with BioDrop DUO UV/Vis spectrophotometer (BioDrop, UK). Successfully extracted genomic DNA was directly used as

template for PCR reactions.

The 16S rRNA gene was sequenced to identify bacterial isolates. All reactions were conducted using universal primers 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') (IDT Inc., Singapore), 1492R (5'-GGT TAC CTT GTT ACG ACT T-3') (IDT Inc., Singapore) and KAPA HiFi™ PCR Kit (Kapa Biosystems, USA) in 50 µl total volume per reaction. The 16S rRNA PCR product was sent to 1st BASE Laboratories Pte. Ltd., Malaysia, for sequencing. The 16S rRNA gene partial sequences of selective isolates were processed using Sequence Scanner 2 (Applied Biosystems, USA) and BioEdit software (Ibis Therapeutics, USA). The acquired 16S rRNA sequences of the isolates were aligned with NCBI GenBank database using BLAST algorithm (<http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi>) for comparison to previously deposited 16S rRNA sequences.

Antimicrobial activity

All representative isolates were tested for the assessment of antimicrobial ability. Antimicrobial effect of isolates was evaluated by well diffusion test on Nutrient Agar (NA; Merck, Germany) plated with two pathogens. For this purpose, fresh culture of isolates were centrifuged (2,400 ×g, 3 min) and 50 µl supernatants of each isolates were added into separate well in NA medium spread plated with *S. aureus* and *E. coli* bacteria standard BTCC (Biotechnology Culture Collection). Development of inhibition zones of both pathogens by supernatant of the tested isolates were assessed after incubation at 37°C for 24 h.

Plasmid extraction

A pure colony of each representative isolates and +pGLO *E. coli* cells (S2) were cultured into 5 ml of growth media and incubated microaerobically at 37°C overnight. The culture was then centrifuged for 2 min at 16,000 ×g. The pellets obtained were subjected to plasmid DNA extraction/purification using Wizard® Plus SV Minipreps DNA Purification System (Promega, USA) as according to the manufacturer's instructions. Extracted plasmids were loaded on a 0.8% agarose gel and electrophoresed for 20 min at 100 V. Plasmid DNA bands were visualized under UV transilluminator (Uvitec, UK).

Antibiotic susceptibility test

Antibiotic profiling was done as described in CLSI guideline using disc diffusion method as described by Liofilchem manual [26]. Selected isolates were examined against 22 antibiotics. Pure cultures were inoculated in MRSB at 37°C overnight and the turbidity was adjusted to 1.0 McFarland standard in 0.85% (wt/vol) sterile saline medium. The suspension was swabbed plated onto MRSA using a sterile cotton swab. Antimicrobial discs were placed on the surface of the agar plates and were incubated microaerobically at 37°C for 24 h. Antimicrobial disc employed include inhibitors of protein synthesis (tiamulin, erythromycin, tylosin, kanamycin, neomycin, streptomycin, gentamicin, chloramphenicol, lincomycin, clindamycin, and tetracycline), cell wall synthesis inhibitor (amoxicillin, ampicillin, vancomycin, oxacillin, cefoxitin), inhibitor of folic acid synthesis (sulfonamide), RNA polymerase inhibitor (rifampicin), DNA gyrase inhibitor (nalidixic acid, ofloxacin, and ciprofloxacin), and cell wall and protein synthesis inhibitor (bacitracin). The antibiotics were applied in form of discs (Liofilchem, Italy) [27].

Starter culture preparation and application

The obtained *L. delbrueckii* and *S. thermophilus* were made into starter culture to ferment full-cream milk for yogurt production as described by Tzia and Sfakianakis [28]. The growth of *L. delbrueckii* and *S. thermophilus* in liquid media was assessed *via* hourly time series measurements through optical density at wavelength 600 nm (OD₆₀₀) and was plated on MRSA media at stationary phase for CFU counts. For each isolates, dilutions were made to achieve 10⁷–10⁹ CFU/ml and were centrifuged at 2,400 ×g for 3 min to obtain a media-free cell pellet before added into full-cream milk. Fermentation process was conducted at 37°C on the first 4 h followed by 42°C for the next 5.5 h in a tightly covered container. Yogurt was stored at 4°C for later consumption. To ensure the quality of yogurt, pH of yogurt was assessed to fall into range of pH 4.0–4.6 after fermentation. Yogurt texture, fermented odor, finished flavor, and appearance were evaluated by volunteers' ratings presented on a 7-point scale ranging from 7 ("extremely like") to 1 ("extremely dislike"). Results were compared to commercial yogurt made from commercial starter culture [29].

Table 1. Selection criteria of forty-nine isolates with similar profiles to *Lactobacillus* genera under different temperature and salinity concentration.

Isolates code	Growth at different temperatures		Growth at different NaCl concentration		
	10 °C	45 °C	2%	4%	5%
89, 163 & 183	(+)	(+)	(+)	(+)	(-)
119	(+)	(-)	(+)	(+)	(+)
109 & 113	(+)	(-)	(-)	(-)	(+)
102, 108, 110, 133 & 184	(+)	(+)	(+)	(+)	(+)
80, 121 & 122	(-)	(-)	(+)	(+)	(-)
60 & 81	(-)	(-)	(+)	(-)	(-)
20, 67, 82, 83, 85, 106, 120 & 137	(-)	(-)	(+)	(+)	(+)
129	(-)	(+)	(+)	(-)	(-)
101	(-)	(+)	(+)	(-)	(+)
92, 126, 161 & 169	(-)	(+)	(+)	(+)	(+)
38, 68, 94, 127, 131 & 132	(-)	(+)	(+)	(+)	(-)*
19, 23, 28, 30, 34, 87, 88, 90, 97, 105, 107, 130 & 162	(-)	(+)	(+)	(+)	(-)

Forty-nine isolates were subjected to grow on MRSB incubated at 45 °C and 10 °C; NaCl concentration at 2%, 4% and 5% [25]. (+) = growth not affected under selected condition, (-) = no growth under selected condition, (-)* = weak growth. Growth was measured by spectrophotometry reading. The strain *Lactobacillus delbrueckii* profile is (-) 10 °C, (+) 45 °C, (+) 2% and 4% and (-) at NaCl ≥ 5%. Shaded region denote unmatched profile for isolate exclusion.

Results and Discussion

Isolation and identification of *Lactobacillus delbrueckii* and *Streptococcus thermophilus*

Referencing to Bergey's manual [25], a total of 49 isolates out of 204 cultured colonies were identified to match general biochemical characteristics of *Lactobacillus* sp., and 10 isolates to match *Streptococcus* sp.. *L. delbrueckii* is known to be able to grow well on 45 °C but not 10 °C, and also to grow at NaCl concentration of 2% and 4%. Growth of *L. delbrueckii* will be affected at NaCl concentration above 5%. *S. thermophilus* is able to grow

on 45 °C but not 10 °C, and at NaCl concentration of 2% but not 4%. The potential of isolates to grow on selected conditions are summarized in Table 1 and 2.

It was assumed that out of matched biochemical characteristics, only 19 isolates displayed similar characteristic as representatives after *L. delbrueckii* and 2 isolates after *S. thermophilus*. The classification of general biochemical together with the available sugar fermenting profile (mono/poly-saccharides and sugar-alcohol) of *L. delbrueckii* no doubt overlaps with other species in the *Lactobacillus* spp. (Table 3A). Colony morphology evaluation enables the isolated *Lactobacillus*

Table 2. Growth selection of ten isolates with similar profiles to *S. thermophilus* to grow at different temperature and salinity concentration.

Isolates code	Growth at different temperature		Growth at different NaCl concentration		
	10 °C	45 °C	2%	4%	5%
4, 16, 77, 99, 170	(-)	(-)	(+)	(-)	(-)
66, 148	(-)	(-)	(+)	(+)	(-)
143	(-)	(-)	(+)	(+)	(+)
24, 98	(-)	(+)	(+)	(-)	(-)

Ten isolates were subjected to grow on MRSB incubated at 45 °C and 10 °C; NaCl concentration at 2%, 4% and 5% [25]. (+) = growth not affected under selected condition, (-) = no growth under selected condition. The strain *S. thermophilus* profile is (-) 10 °C, (+) 45 °C, (+) 2% and (-) at NaCl ≥ 4%. Shaded region denote unmatched profile for isolate exclusion.

Table 3. Classification of isolates based on sugar-fermenting ability, colony morphology and molecular species identity.

			Representative Isolate/Isolate Group				
			24/S1	107/L1	87/L2	97/L3	94/L4
A. Types of Sugar	(Mono) saccharide	Glucose	(+)	(+)	(+)	(+)	(+)
		Fructose	(+)	(+)	(+)	(+)	(+)
	(Di-) saccharide	Lactose	(+)	(+)	(+)	(+)	(+)
		Maltose	(+)	(+)	(+)	(+)	(+)
		Sucrose	(+)	(+)	(+)	(+)	(+)
	(Tri-) saccharide	Raffinose	(+)	(+)	(+)	(+)	(+)
	Sugar - Alcohol	Mannitol	(-)	(-)	(+)	(+)	(+)
		Sorbitol	(+)	(+)	(+)	(+)	(+)
B. Colony Morphology	Shape	Circular			Circular		
	Margin	Entire			Entire		
	Texture	Smooth			Smooth		
	Pigmentation	Non-Pigmented			Non Pigmented		
	Optical Property	Opaque			Opaque		
	Elevation	Flat		Flat	Pulvinate		
	Appearance	Glistening		Glistening	Dull		
	Size	Small	Small	Moderate	Small	Moderate	
C. Species Identity (16S rRNA)			<i>S. thermophilus</i>	<i>L. fermentum</i>	<i>L. fermentum</i>	<i>L. casei</i>	<i>L. delbrueckii</i>

A. The ability of isolates to ferment different types of sugars; B. Classification of isolates based on colony morphologies; C. Species identity after 16S rRNA homology search. (+) = positive sugar fermenter, (-) = negative sugar fermenter. Isolate Group S1: isolate 24 and 98; Isolate Group L1: isolate 107; Isolate Group L2: isolate 19, 23, 28, 30, 34, 87, 88, 90, 105, 130 and 162; Isolate Group L3: isolate 97; and Isolate Group L4: isolate 38, 68, 94, 127, 131 and 132.

spp. to be classified into 4 groups of *Lactobacillus* spp. (L1-4) and 1 group of *S. thermophilus* (Table 3B). 16S rRNA sequencing revealed that a representative of 1 group (L4) was identified to be *L. delbrueckii*, represented by *L. delbrueckii* 94/L4 and another representative of 1 group (S1) was identified to be *S. thermophilus*, represented by *S. thermophilus* 24/S1. Other *Lactobacillus* species recognized after 16S rRNA sequencing were *L. fermentum* (107/L1 and 87/L2) and *L. casei* (97/L3). Nevertheless, the isolation of other *Lactobacillus* species might be useful to give a glimpse idea on the assortment of LAB biodiversity that exist in the indigenous fresh milk.

Obtained 16S rRNA sequences of representative isolates are available in GenBank under accession number MH298532 (*S. thermophilus* 24/S1), MH298578 (*L. fermentum* 107/L1), MH348995 (*L. fermentum* 87/L2), MH298536 (*L. casei* 97/L3) and MH298535 (*L. delbrueckii* 94/L4). The method proposed by Bergey's manual provides a satisfactory accuracy with regard to successful isolation of *S. thermophilus* and *L. delbrueckii*.

Antimicrobial activity of *Lactobacillus* spp. and *S. thermophilus* 24/S1

The selected isolates *S. thermophilus* 24/S1, *L. fermentum* 107/L1, *L. fermentum* 87/L2, *L. casei* 97/L3 and *L. delbrueckii* 94/L4 were examined after their antimicrobial activity against Gram-negative model pathogen *E. coli* and Gram-positive model pathogen *S. aureus*. The recorded inhibition zone diameter for tested *Lactobacillus* strains ranged from 14.00 ± 2.08 mm (*L. fermentum* 87/L2) to 19.00 ± 1.00 mm (*L. delbrueckii* 94/L4) against *E. coli* and from 11.00 ± 0.00 mm (*L. delbrueckii* 94/L4) to 15.50 mm ± 0.00 (*L. fermentum* 87/L2) against Gram-positive *S. aureus*.

The *Lactobacillus* strains 94/L4, representing *L. delbrueckii* group, had a high antimicrobial activity against the *E. coli* (19.00 ± 1.00) and low antimicrobial activity against *S. aureus* (11.00 ± 0.00). The *S. thermophilus* 24/S1 had the lowest antimicrobial activity against all tested model pathogens (12.67 ± 2.08 against *E. coli* and 14.00 ± 1.73 against *S. aureus*) (Table 4 and S1). According to previous findings, it was reported that

Table 4. Antimicrobial activity of *S. thermophilus* 24/S1, *L. fermentum* 107/L1, *L. fermentum* 87/L2, *L. casei* 97/L3, *L. delbrueckii* 94/L4 against Gram-negative and Gram-positive model pathogen.

Representative isolates/control	Disc inhibition zone (mm)	
	<i>E. coli</i>	<i>S. aureus</i>
(-)	0	0
(+)	32.00 ± 1.53	36.50 ± 1.73
<i>S. thermophilus</i> 24/S1	12.67 ± 2.08	14.00 ± 1.73
<i>L. fermentum</i> 107/L1	17.67 ± 1.15	14.67 ± 0.58
<i>L. fermentum</i> 87/L2	14.00 ± 2.08	15.5 ± 0.71
<i>L. casei</i> 97/L3	18.67 ± 1.15	15.00 ± 0.00
<i>L. delbrueckii</i> 94/L4	19.00 ± 1.00	11.00 ± 0.00

Values are given as Mean ± SD. (-): MRSB (Negative control); (+): Ampicillin at 5 µg per well (Positive control).

L. delbrueckii strains exhibited antimicrobial activity and able to inhibit *E. coli* infection [30, 31] while *S. thermophilus* T2 strain showed antimicrobial activity against the Gram-positive bacteria [32]. The variable inhibitory action of all tested isolates towards *E. coli* and *S. aureus* may apparently be associated to their ability to secrete different extend of antibacterial substances including lactic acid, hydrogen peroxide or bacteriocin [33]. The production of bacteriocin by most of the genera of LAB, including *L. delbrueckii* (i.e. bulgarican) has been substantially reported [34, 35].

Antibiotic susceptibility test of *S. thermophilus* 24/S1 and *L. delbrueckii* 94/L4

Before *S. thermophilus* 24/S1 and *L. delbrueckii* 94/L4 were subjected to antibiotic susceptibility test, both isolates was first verified for any presence of plasmid to ensure their characteristic was not depicted through the influence of plasmid. Plasmid was not detected after extraction (S2 and S3). The presence of non-transferrable resistance gene intrinsically will be valuable upon exposure to particular antibiotics asserting their safety and beneficial effect on host.

Antibiotic profiling performed as indicated on CLSI guideline [26] by disc diffusion method showed that both tested isolates demonstrate potential resistance against all tested antibiotic with DNA gyrase inhibition as mode of action (ofloxacin, ciprofloxacin and nalidixic acid) and obstruction through folic acid pathway (sulphonamide); and susceptible against rifampicin and bacitracin with

Table 5. Antibiotic resistance profiles of *L. delbrueckii* 94/L4 and *S. thermophilus* 24/S1.

Mode of action	Antibiotic (µg)	<i>L. delbrueckii</i> 94/L4	<i>S. thermophilus</i> 24/S1
DNA Gyrase Inhibition	OFX (5)	R	R
	CIP (5)	R	R
	NA (30)	R	R
Folic Acid Pathway Inhibition	SUL (300)	R	R
	RD (5)	S	S
Cell Wall and Protein Synthesis Inhibition	BA (10)	S	S
	AML (20)	S	S
	AMP (2)	S	S
	VA (30)	R	R
	OX (1)	R	S
Protein Synthesis Inhibition	FOX(30)	R	I
	MY (2)	S	R
	T (30)	S	R
	E (15)	S	R
	CD (2)	S	R
	TE (30)	S	R
	TY (30)	S	R
	K (30)	R	S
	S (10)	R	S
	CN (10)	R	R
N (30)	R	R	
C (30)	S	R	

OFX, ofloxacin; CIP, ciprofloxacin; NA: nalidixic acid, SUL: sulphonamide; RD: rifampicin; BA: bacitracin; AML: amoxicillin; AMP: ampicillin; VA: vancomycin; OX: oxacillin; FOX: ceftioxin; MY: lincomycin; T: tiamulin; E: erythromycin; CD: clindamycin; TE: tetracycline; TY: tylosin; K: kanamycin; S: streptomycin; CN: gentamicin; N: neomycin; C: chloramphenicol. S: susceptible, I: intermediate, R: resistance.

RNA polymerase and cell wall/protein synthesis inhibition mode of action, respectively. *S. thermophilus* 24/S1 and *L. delbrueckii* 94/L4 showed to be resistant to a range of antibiotics. Both isolates have different antibiotic susceptibility profiles displaying similar susceptibility against the most commonly prescribed antibiotics, amoxicillin and appears to be resistant against sulphonamide (Table 5, S4–6) [36].

Growth of the LAB was conducted on MRSA instead of Muller Hinton (MH) agar. Growth of *Lactobacillus* on

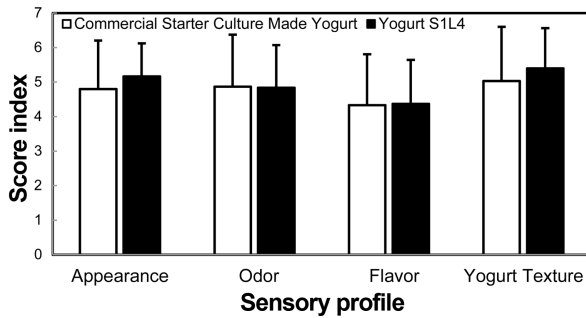


Fig. 1. The sensory evaluation of yogurt given by volunteers using selected culture (Yogurt S1L4) and commercial starter cultures. Score index is ranging from 1-7. Score of 1 = "extreme dislike", and 7 = "extreme like" were given on the appearance, fermented odor, finished flavor and texture. Total samples (n) = 30. Error bar represents SD.

MH agar was known to be poorly produced undefined inhibition zone [37]. The growth of the selected LAB *S. thermophilus* 24/S1 and *L. delbrueckii* 94/L4 on MRSA was homogeneous and the inhibition halos were clearly distinctive though generally larger zone was produced as compared to other media, such as LAPTg and MH agar, therefore McFarland turbidity was adjusted to 1.0 to give a more conveniently distinctive background for inhibition zone diameter measurement [38]. There are currently no available antibiotic disc diffusion breakpoints standard for *L. delbrueckii* and *S. thermophilus*, therefore susceptible and resistance range data reflected by inhibition zone diameter obtained across all species as listed in Performance Standards for Antimicrobial Susceptibility Testing (M100-S24) provided by Clinical and Laboratory Standards Institute (CLSI) were adopted for this test as breakpoints reference for disc diffusion antibiotic test [39, 40].

Generally, consistent with our findings, *Lactobacillus* has been reported to be resistant to kanamycin, gentamycin and streptomycin. It is also reported to be intrinsically resistant to vancomycin [41, 42] by replacing the terminal D-alanine by D-lactate or D-serine in the pentapeptide of the peptidoglycan [43]. *Lactobacillus* sensitivity to rifampicin, tetracycline, erythromycin and clindamycin and cell wall synthesis inhibitors such as penicillin and ampicillin has also been reported [44, 45]. *S. thermophilus* strains were previously described to have acquired resistance gene responsible for their resistance to erythromycin, streptomycin and tetracycline

[45]. Despite being non-pathogenic, *S. thermophilus* 24/S1 and *L. delbrueckii* 94/L4 should be subjected to genome analysis to further confirm the nature of its antibiotic resistance to avoid the chance on the existence of transferrable gene.

***S. thermophilus* 24/S1 and *L. delbrueckii* 94/L4 as potential starter culture for yogurt production**

Growth curve of *S. thermophilus* 24/S1 and *L. delbrueckii* 94/L4 were evaluated (S7). Both probiotic for starter culture were grown to stationary phase before inoculated into fresh milk. Yogurt S1L4 was made and compared to the product of commercial starter culture through the qualities of yogurt samples. The making of yogurt S1L4 took a total 9.5 h of incubation, 30 minutes longer than the commercial starter culture [29]. The stated incubation time taken was consistent with reproducible quality. Final acidity of yogurt S1L4 made and commercial yogurt are pH 4.42 and pH 4.58, respectively. Initial sensory profile was made through comparing yogurt S1L4 with yogurt made from commercial starter culture, which contains similar probiotic content (*S. thermophilus* and *L. delbrueckii*) with the addition of *L. acidophilus* and *Bifidobacterium* sp. BB-12 [29].

The potential *L. delbrueckii* 94/L4 and *S. thermophilus* 24/S1 as starter culture made into food product was assayed by recruiting 30 non-expert volunteers and yield satisfactory response through feedbacks obtained from 30 volunteers. Score index of yogurt S1L4 from the tested starter culture (4.93 ± 0.45) was similar to the yogurt made from commercial starter cultures (4.76 ± 0.30) with a better score on the appearance (5.16 ± 0.95 and 4.80 ± 1.40), finished flavor (4.36 ± 1.27 and 4.33 ± 1.47), texture (5.40 ± 1.17 and 5.03 ± 1.56), except for the fermented odor (4.83 ± 1.23 and 4.86 ± 1.50) which was a little lower than commercial starters (Fig. 1).

Starter culture S1L4 consisting of our own isolated *S. thermophilus* 24/S1 and *L. delbrueckii* 94/L4 from indigenous fresh milk has successfully been assimilated on the fermentation of yogurt S1L4 with comparable taste quality to yogurt made by commercial starters containing additional probiotic species (*L. acidophilus* and *Bifidobacterium* sp. BB-12). Despite being non-pathogenic, *S. thermophilus* 24/S1 and *Lactobacillus* strains 94/L4 should be further verified on its detailed characteristics on benefits and safety through genome analysis before

applying the isolates commercially. Organoleptic assays should be conducted through the recruitment of experts to further verify the quality of the food product made.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

References

- Wagner RD, Johnson SJ. 2017. Probiotic bacteria prevent *Salmonella* – induced suppression of lymphoproliferation in mice by an immunomodulatory mechanism. *BMC Microbiol.* **17**: 77.
- Abdel-Daim A, Hassouna N, Hafez M, Ashor MSA, Aboulwafa MM. 2013. Antagonistic activity of *Lactobacillus* isolates against *Salmonella typhi* in vitro. *Biomed. Res. Int.* **2013**: 680605.
- Cordonnier C, Thévenot J, Etienne-Mesmin L, Alric M, Livrelli V, Blanquet-Diot S. 2017. Probiotic and enterohemorrhagic *Escherichia coli*: An effective strategy against a deadly enemy? *Crit. Rev. Microbiol.* **43**: 116-132.
- Licciardi PV, Toh ZQ, Dunne E, Wong S-S, Mulholland EK, Tang M. et al. 2012. Protecting against pneumococcal disease: Critical interactions between probiotics and the airway microbiome. *PLoS Pathog.* **8**: e1002652.
- Mogna L, Deidda F, Nicola S, Amoroso A, Del Piano M, Mogna G. 2016. In Vitro inhibition of *Klebsiella pneumoniae* by *Lactobacillus delbrueckii* subsp. *delbrueckii* LDD01 (DSM 22106): An innovative strategy to possibly counteract such infections in humans? *J. Clin. Gastroenterol.* **50**: S136-S139.
- Ngwa CJ, Pradel G. 2015. Coming soon: probiotics-based malaria vaccines. *Trends Parasitol.* **31**: 2-4.
- Shi LH, Balakrishnan K, Thiagarajah K, Ismail NIM, Yin OS. 2016. Beneficial properties of probiotics. *Trop. Life Sci. Res.* **27**: 73-90.
- Ouwehand AC, Forssten S, Hibberd AA, Lyra A, Stahl B. 2016. Probiotic approach to prevent antibiotic resistance. *Ann. Med.* **48**: 246-255.
- Krzych-Falta E, Furmańczyk K, Tomaszewska A, Olejniczak D, Samoliński B, Samolińska-Zawisza U. 2018. Probiotics: Myths or facts about their role in allergy prevention. *Adv. Clin. Exp. Med.* **27**: 119-124.
- Chaucheyras-Durand F, Durand H. 2010. Probiotics in animal nutrition and health. *Benef. Microbes.* **1**: 3-9.
- Agaliya PJ, Jeevaratnam K. 2013. Molecular characterization of lactobacilli isolated from fermented *idli* batter. *Braz. J. Microbiol.* **44**: 1199-1206.
- Verso LL, Lessard M, Talbot G, Fernandez B, Fliss I. 2017. Isolation and selection of potential probiotic bacteria from the pig gastrointestinal tract. *Probiotics Antimicrob. Proteins.* **10**: 299-312.
- Yun JH, Lee KB, Sung YK, Kim EB, Lee HG, Choi YJ. 2009. Isolation and characterization of potential probiotic lactobacilli from pig feces. *J. Basic Microbiol.* **49**: 220-226.
- Matei G-M, Matei S, Mocanu V. 2016. Isolation of new probiotic microorganisms from soil and screening for their antimicrobial activity. *Soil Forming Factors and Processes from the Temperate Zone.* **15**: 21-26.
- Zoumpoulou G, Tzouvanou A, Mavrogonatou E, Alexandraki V, Georgalaki M, Anastasiou R. et al. 2017. Probiotic features of lactic acid bacteria isolated from a diverse pool of traditional Greek dairy products regarding specific strain-host interactions. *Probiotics Antimicrob. Proteins.* **10**: 313-322.
- Kechagia M, Basoulis D, Konstantopoulou S, Dimitriadi D, Gyftopoulou K, Skarmoutsou N. et al. 2013. Health benefits of probiotics: A review. *ISRN Nutr.* **2013**: 481651.
- Fijan S. 2014. Microorganisms with claimed probiotic properties: An overview of recent literature. *Int. J. Environ. Res. Public Health.* **11**: 4745-4767.
- Quigley L, O'Sullivan O, Stanton C, Beresford TP, Ross RP, Fitzgerald GF. et al. 2013. The complex microbiota of raw milk. *FEMS Microbiol. Rev.* **37**: 664-698.
- Iyer R, Tomar SK, Maheswari TU, Singh R. 2010. *Streptococcus thermophilus* strains: Multifunctional lactic acid bacteria. *Int. Dairy J.* **20**: 133-141.
- Yerlikaya O. 2014. Starter cultures used in probiotic dairy product preparation and popular probiotic dairy drinks. *Food Sci. Technol. (Campinas)* **34**: 221-229.
- Rul F, Ben-Yahia L, Chegdani F, Wrzosek L, Thomas S, Noordine ML. et al. 2011. Impact of the metabolic activity of *Streptococcus thermophilus* on the colon epithelium of gnotobiotic rats. *J. Biol. Chem.* **286**: 10288-10296.
- Labayen I, Forga L, González A, Lenoir-Wijnkoop I, Nutr R, Martínez JA. 2001. Relationship between lactose digestion, gastrointestinal transit time and symptoms in lactose malabsorbers after dairy consumption. *Aliment. Pharmacol. Ther.* **15**: 543-549.
- Surono IS. 2015. Traditional Indonesian dairy foods. *Asia Pac. J. Clin. Nutr.* **24**: S26-S30.
- Grand View Research. (n.d.). Starter Culture Market Analysis, Market Size, Application Analysis, Regional Outlook, Competitive Strategies And Forecasts, 2014 To 2020. Available from <https://www.grandviewresearch.com/industry-analysis/starter-culture-market>. Accessed May 7, 2018.
- Vos P, Garrity G, Jones D, Krieg NR, Ludwig W, Rainey FA, Schleifer K-H, Whitman W (eds.). 2009. *Bergey's Manual of Systemic Bacteriology Volume 3: The Firmicutes*, pp. 253, 465-532. 2nd Ed. Springer-Verlag New York, New York.
- Clinical and Laboratory Standards Institute. 2006. *M45 Methods for antimicrobial dilution and disk susceptibility testing of infre-*

- quently isolated or fastidious bacteria. 3rd Ed. Clinical and Laboratory Standards Institute, Wayne, Pennsylvania.
27. Liofilchem. 2017. Antibiotic Disc. Available from <http://www.liofilchem.net/login/pd/pi/AD.pdf>. Accessed May 7, 2018.
 28. Sfakianakis P, Tzia C. 2014. Conventional and innovative processing of milk for yogurt manufacture; development of texture and flavor: A Review. *Foods*. **3**: 176-193.
 29. Chr. Hansen. 2018. Culture range for fermented milk. Available from https://hjemmeriet.com/da/ChrHansen/Brochures/Fermented_milk_range_brochure.pdf. Accessed May 7, 2018.
 30. Akpınar A, Yerlikaya O, Kiliç S. 2011. Antimicrobial activity and antibiotic resistance of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* strains isolated from Turkish homemade yoghurts. *Afr. J. Microbiol. Res.* **5**: 675-682.
 31. Abedi D, Feizizadeh S, Akbari V, Jafarian-Dehkordi A. 2013. *In vitro* anti-bacterial and anti-adherence effects of *Lactobacillus delbrueckii* subsp *bulgaricus* on *Escherichia coli*. *Res. Pharm. Sci.* **8**: 260-268.
 32. Mezaini A, Chihib N-E, Bouras AD, Nedjar-Arroume N, Hornez JP. 2009. Antibacterial activity of some lactic acid bacteria isolated from an Algerian dairy product. *J. Environ. Public. Health.* **2009**: 678495.
 33. Gibbs PA. 1987. Novel uses for lactic acid fermentation in food preservation. *J. Appl. Microbiol.* **63**: 515-585.
 34. Simova ED, Beshkova DM, Angelov MP, Dimitrov ZhP. 2008. Bacteriocin production by strain *Lactobacillus delbrueckii* ssp. *bulgaricus* BB18 during continuous prefermentation of yogurt starter culture and subsequent batch coagulation of milk. *J. Ind. Microbiol. Biotechnol.* **35**: 559-567.
 35. Todorov SD. 2009. Bacteriocins from *Lactobacillus plantarum* - production, genetic organization and mode of action: produção, organização genética e modo de ação. *Braz. J. Microbiol.* **40**: 209-221.
 36. Pradipta IS, Ronasih E, Kartikawati AD, Hartanto H, Amelia R, Febrina E. et al. 2015. Three years of antibacterial consumption in Indonesian Community Health Centers: The application of anatomical therapeutic chemical/defined daily doses and drug utilization 90% method to monitor antibacterial use. *J. Family Commun. Med.* **22**: 101-105.
 37. Ocaña V, Silva C, Nader-Macías ME. 2006. Antibiotic susceptibility of potentially probiotic vaginal lactobacilli. *Infect. Dis. Obstet. Gynecol.* **2006**: 18182.
 38. Huys G, D'Haene K, Swings J. 2002. Influence of the culture medium on antibiotic susceptibility testing of food-associated lactic acid bacteria with the agar overlay disc diffusion method. *Lett. Appl. Microbiol.* **34**: 402-406.
 39. Clinical and Laboratory Standards Institute. 2014. *M100-S24 Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fourth Informational Supplement*. Clinical and Laboratory Standards Institute, Wayne, Pennsylvania.
 40. Jones RN, Fritsche TR, Sader HS, Ross JE. 2006. Activity of retapamulin (SB-275833), a novel pleuromutilin, against selected resistant Gram-positive cocci. *Antimicrob. Agents Chemother.* **50**: 2583-2586.
 41. Ruoff KL, Kuritzkes DR, Wolfson JS, Ferraro MJ. 1988. Vancomycin-resistant gram-positive bacteria isolated from human sources. *J. Clin. Microbiol.* **26**: 2064-2068.
 42. Zhou JS, Pillidge CJ, Gopal PK, Gill HS. 2005. Antibiotic susceptibility profiles of new probiotic *Lactobacillus* and *Bifidobacterium* strains. *Int. J. Food Microbiol.* **98**: 211-217.
 43. Delcour J, Ferain T, Deghorain M, Palumbo E, Hols P. 1999. The biosynthesis and functionality of the cell-wall of lactic acid bacteria. *Antonie Van Leeuwenhoek.* **76**: 159-184.
 44. Kyriacou A, Tsimpidi E, Kazantzi E, Mitsou E, Kirtzalidou E, Oikonomou Y. et al. 2008. Microbial content and antibiotic susceptibility of bacterial isolates from yoghurts. *Int. J. Food Sci. Nutr.* **59**: 512-525.
 45. Hummel AS, Hertel C, Holzapfel WH, Franz CMAP. 2007. Antibiotic resistances of starter and probiotic strains of lactic acid bacteria. *Appl. Environ. Microbiol.* **73**: 730-739.