International Journal on Advanced Science Engineering Information Technology

Vol.9 (2019) No. 5
ISSN: 2088-5334

Characterization of Probiotic Bacterial Candidates from Jatinangor-Indonesia Breast Milk

In-In Hanidah[#], Indira Lanti Kaya Putri[#], Wendry Setiyadi Putranto[*], Bambang Nurhadi[#], Debby Moody Sumanti[#]

[#]*Food Industrial Technology Department, Faculty of Agricultural Industrial Technology, Universitas Padjadjaran, 45363 Sumedang, Indonesia.*
Email: inin@unpad.ac.id

[*]*Laboratory of Animal Products Technology, Faculty of Animal Husbandry, Universitas Padjadjaran, 45363 Sumedang, Indonesia.*

Abstract*— Breast milk is an important nutrient for neonates in body's nutritional needs and immune system formation. Some research show breast milk generally contains Bifidobacteria and probiotic bacteria species of Lactobacillus. Microbiota in the breast milk from every area is diverse, bacteria isolated from healthy mothers' breast milk from Taiwan and six regions of China (Central, East, North, Northeast, South, and Southwest China). It shows Streptococcaceae (24.4%), Pseudomonadaceae (14.0%), Staphylococcaceae (12.2%), Lactobacillaceae (6.2%), and Oxalobacteraceae (4.8%). Germany or Austria provided the breast milk from 160 women contain L. salivarius (35.00%), L. fermentum (25.00%), L. gasseri (21.88%), and Bifidobacterial species (13.75%). Spanish provided Staphylococcus, Pseudomonas, Streptococcus, and Acinetobacter dominated the breast milk from 21 healthy mother. In this study, isolation and characterization of candidates for probiotic bacteria from fifteen breast milk samples from Sumedang – Indonesia. The main purpose of this study was to isolate and identify probiotic bacteria that are resistant pH 2 and tolerance of 0.3% bile concentration. The results showed that were twelve isolate candidate probiotic bacteria able to grow on pH 2 media for 2 hours and tolerance of 0.3% bile concentration. Only two of them had the best growth and potential probiotic candidates. Base on Biochemical identification using the Vitek 2.0 Card type: ANC testing instrument 00001658F4A9 (12903), there are Staphylococcus hominis (8.3%) and Lactobacillus plantarum (8.3%). In the future, *S. hominis* will be probiotic bacteria that can be applied as functional food.

***Keywords*— breast milk; probiotic; *Staphylococcus hominis*; *Lactobacillus plantarum;* functional food.**

I. INTRODUCTION

Breast milk is a perfect nutrient for infants [1] and potentially probiotic bacteria to the infant gut [2]. It is among others: immunology, biochemical component (proteins, lipids, carbohydrates, biological active), and cellular component that are very potential for the newborn's immune system from various infections [3]. These components are very important for infants and it transfers microflora originated in breast milk [4]. Breast milk is also proven to be a source of commensal and probiotic bacteria such as *Staphylococcus*, *Streptococcus*, and Lactic Acid Bacteria (LAB) [5].

Some research show breast milk generally contains Bifidobatreria [6] and probiotic bacteria species of Lactobacillus [7]. Microbiota in the breast milk from every area is diverse, bacteria isolated from healthy mothers' breast milk from Taiwan and six regions of China (Central, East, North, Northeast, South, and Southwest China). It shows Streptococcaceae (24.4%), Pseudomonadaceae (14.0%), Staphylococcaceae (12.2%), Lactobacillaceae (6.2%), and Oxalobacteraceae (4.8%) [8]. Germany or Austria provided the breast milk from 160 women contain L. salivarius (35.00%), L. fermentum (25.00%) and L. gasseri (21.88%), and Bifidobacterial species (13.75%) [6]. Spanish provided the breast milk from 21 healthy mother was dominated by *Staphylococcus*, *Pseudomonas*, *Streptococcus*, and *Acinetobacter*[9]. In another Spanish, the healthy core microbiome included the genera *Staphylococcus*, *Streptococcus*, *Bacteroides*, *Faecalibacterium*, *Ruminococcus*, *Lactobacillus*, and *Propionibacterium* [10].

Although probiotic strains can be isolated from many sources, but for human applications the main criteria is being human origin [11]. Geographical location can directly affect the microbiota and fatty acid content in breast milk[8]. Milk bacterial communities were generally complex and showed individual specific profiles [9].

II. Material and Method

A. Material

MRS-broth CM0359 oxoid, MRS-Agar (Oxoid CM0361), Hydrochloric acid fuming 37% for analysis Merck K49353217730, bile salts HIMEDIA RM008, UV-9200 Spectrophotometer, Vitek 2.0 (Laboratory of Biofarma) testing instrument 00001658F4A9 (12903), pH meter.

B. Method

1) Subjects and sampling Breast Milk: Sample obtained from 15 healthy women breastfeeding from Jatinangor - Indonesia according to the following criteria: (i) women with healthy history until samples are taken; (ii) normal pregnancy term; (iii) absence of infant and maternal perinatal problems; and (iv) not having mastitis in the breast. All volunteers gave written informed consent to the protocol, which was approved by the Ethical Committee of Medicine Faculty Universitas Padjadjaran No: 883/UN6.C.10/PN/2017. The participants provided samples of breast milk days 50 after birth. The milk samples were collected in a sterile tube by manual expression using sterile gloves. Previously, nipples and mammary areola had been cleaned with 70% alcohol and sterile gauze. All the samples were kept at 4°C until delivery to the laboratory[5], which occurred within 30 minute after collection.

2) Isolation Lactic Acid Bacteria (LAB): LAB was isolated from healthy mother by using de Man Rogosa Sharpe broth (MRS-broth CM0359 oxoid). 1 ml sample was mixed with 10 ml of sterile MRS-broth, homogenized, were incubated anaerobically at 37°C for 24 h. Then 1 ml suspension mixed with 10 ml of sterile MRS-broth pH 2, homogenized, was incubated anaerobically at 37°C for 2 h. Next step 1 ml suspension pour plated aseptically using MRS-Agar (Oxoid CM0361) which were incubated anaerobically at 37°C for 48 h.

3) Identification of the probiotic isolates: The selected isolates were observed colony morphological characterization including shape, margin elevation at the bottom or on the surface of the medium, texture, surface pigmentation, size, and gram staining [12].

4) Resistance pH 2: For identifying the bacterial isolates which could tolerate simulated gut acidic conditions. MRS-broth was adjusted to pH 2.0 and inoculated with one ml of log phase bacterial isolate. The inoculated broth was incubated at 37°C for 120 min [13]. At the interval of 30 min, inoculated measured of absorbance at OD_{620} in UV-9200 Spectrophotometer

5) Bile tolerance: The experiment was applied at this concentration of bile for 4 h. MRS medium containing 0.3% (w/v) bile (HIMEDIA) was inoculated with active cultures (incubated for 16-18 h) [11]. The inoculated broth was incubated at 37°C for 4 h. At the interval of 30 min, inoculated measured of absorbance at OD_{620} in UV-9200 Spectrophotometer.

6) Biochemical characterization: Isolate probiotic candidates followed by biochemical testing using the Vitek 2.0 (Laboratory of Biofarma) testing instrument 00001658F4A9 (12903) to find out the types of microorganisms and species.

III. Result and Discussion

A. Isolation and Identification Candidate of Probiotic

The In this study, we isolated a variety of Total Lactic Acid Bacteria (LAB), resistance pH 2, and tolerance 0,3% bile concentration of human milk from Jatinangor - Indonesia. The result showed that from fifteen samples, only ten as potential of probiotic (Table 1). Probiotic is widely applied to human as well as animal to improving gut health [12] and optimize metabolism and immune system[14]. Characterized probiotic showed resistance to stomach pH (pH 2, 3)[13][15] and tolerance against 0,3% bile concentration[11][16].

TABLE I
Selection Criteria of Fifteen Isolates with Similar Profile Probiotic Candidate

Breast Milk Code	Tolerance		Colony Morphology						GRAM
	pH 2	Against Bile	Shape	Margin	Texture	Pigmentation	Elevation	Size	
A1	+	+	circular	entire	smooth	Non-pigmentation	Flat	Small	+
A2	+	+	circular	entire	smooth	Non-pigmentation	Flat	Big	+
A3	+	+	circular	entire	smooth	Non-pigmentation	Flat	Small	+
A4	+	+	circular	entire	smooth	Non-pigmentation	Flat	Small	+
A5	+	+	circular	entire	smooth	Non-pigmentation	Flat	Small	+
A6	-	-	-	-	-	-	-	-	-
A7	+	+	circular	entire	smooth	Non-pigmentation	Flat	Small	+
A8	+	+	circular	entire	smooth	Non-pigmentation	Flat	Big	+
A9	+	+	circular	entire	smooth	Non-pigmentation	Flat	Medium	+
A10	+	+	circular	entire	smooth	Non-pigmentation	Flat	Medium	+
A11	+	+	circular	entire	smooth	Non-pigmentation	Flat	Medium	+
A12	+	+	circular	entire	smooth	Non-pigmentation	Flat	Small	+
A13	-	-	-	-	-	-	-	-	-
A14	-	-	-	-	-	-	-	-	-
A15	+	+	circular	entire	smooth	Non-pigmentation	Flat	Small	+

The probiotic microorganisms mainly consist of the strains of the genera *Lactobacillus*, *Bifidobacterium*, *Streptococcus* and some *Enterococcus* species [17]. Probiotic showed lactic acid bacteria are characterized by their abilities to ferment carbohydrates into lactic acid in MRS agar [18], gram- positive, non-motile, non-spore forming bacteria, non-pigmented, catalyst negative [19], and microaerophilic to strictly anaerobic [20]. The probiotic microorganisms mainly consist of the strains of the genera *Lactobacillus*, *Bifidobacterium*, *Streptococcus* and some *Enterococcus* species [17]. Probiotic showed lactic acid bacteria are characterized by their abilities to ferment carbohydrates into lactic acid in MRS agar [18], gram-positive, non-motile, non-spore forming bacteria, non-pigmented, catalyst negative [19], and microaerophilic to strictly anaerobic [20].

In Taiwan, China, and Spanish bacteria isolated from breast milk healthy mothers dominated by *Staphylococcus* [8][10]. The strain of *Staphylococcus* showed characterized is coagulase negative, gram positive, catalase producing and facultative anaerobe [21]. Based on the previous literature, isolates samples with codes A1, A2, A3, A4, A5, A7, A8, A9, A10, A11, A12, and A15 will be tested for pH 2 and 0,3% bile salt resistance.

B. pH Resistance

Probiotics candidates have to satisfy ability to survive at the harsh condition pH 2 and bile salts tolerance [21]. Ten selected isolates were tested for resistance low pH. The test was carried out by inserting 1 ml of the suspension of the isolate into different sterile broth oxoid mediums. Each MRS broth medium is conditioned at pH of 2. The suspension is incubated at 37^{o}C in an-aerobic chamber. Growth of bacterial was measured through spectrophotometry reading at wavelength 620 nm (OD_{620}) every 30 minute for 2 hours.

The result showed all isolates that survive in pH 2.0 (Fig. 1) were taken to the next step of testing 0,3% bile concentration. However, isolates with code A1, A2, A3, A11, and A12 showed resistance of pH 2 highest compared to other isolates. Strains selected for use as probiotic bacteria should be able to tolerate acid for at least 90 min [22].

The most important characteristic for selecting probiotic candidates is resistance to acidity of the stomach and bile salts. The digestion processes need 2 – 3 hours starting from food intake by mouth, oral cavity, stomach, to enter the upper intestinal tract, which contain bile. Probiotic bacteria should be resistant lyzozyme in the oral cavity, pH 1,5 - 3,0 in the stomach, and bile salt in the upper intestine [11][16].

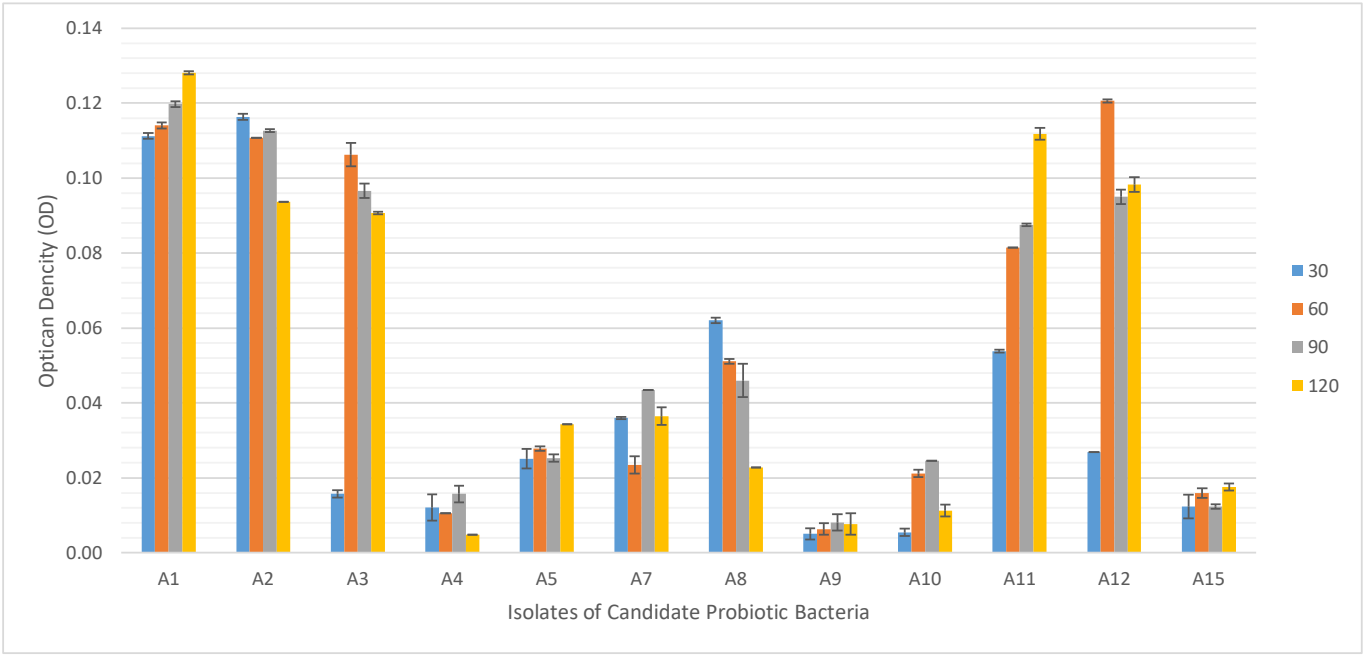

Fig. 1 Growth of candidate isolates of probiotic bacteria that survival in pH 2.0. Measurement based on OD620 values 30 minutes for 2 hours. Error bab represents SD.

C. Resistance to Bile Salt

The results showed that all isolates were able to grow in 0,3% bile concentration in 4 h (240 minutes). The growth of each isolate is different depending on the tolerance of the bacterial isolate to bile. Four out of ten probiotic candidate isolates (A1, A2, A8, and A11) showed good growth for 4 h (Fig. 2).

Bile tolerance are other important characteristics of probiotic lactic acid bacteria enable to survive, to grow and to perform beneficial action in the gastrointestinal tract [20]. Because the mean intestinal bile concentration is believed to be 0.3% (w/v) and the staying time of food in small intestine is suggested to be 4 h [11]. Ten isolates of probiotics candidate were carried out further tests with 0.3% bile salt concentration. The test was carried out by inserting 1 ml of the isolate suspension into a sterile MRS broth media, which had added 0.3% bile salt HIMEDIA. The suspension is incubated at 37^{o}C in an-aerobic chamber. Bacterial growth was measured through spectrophotometric readings at a wavelength of 620 nm (OD_{620}) every 30 minutes for 4 hours.

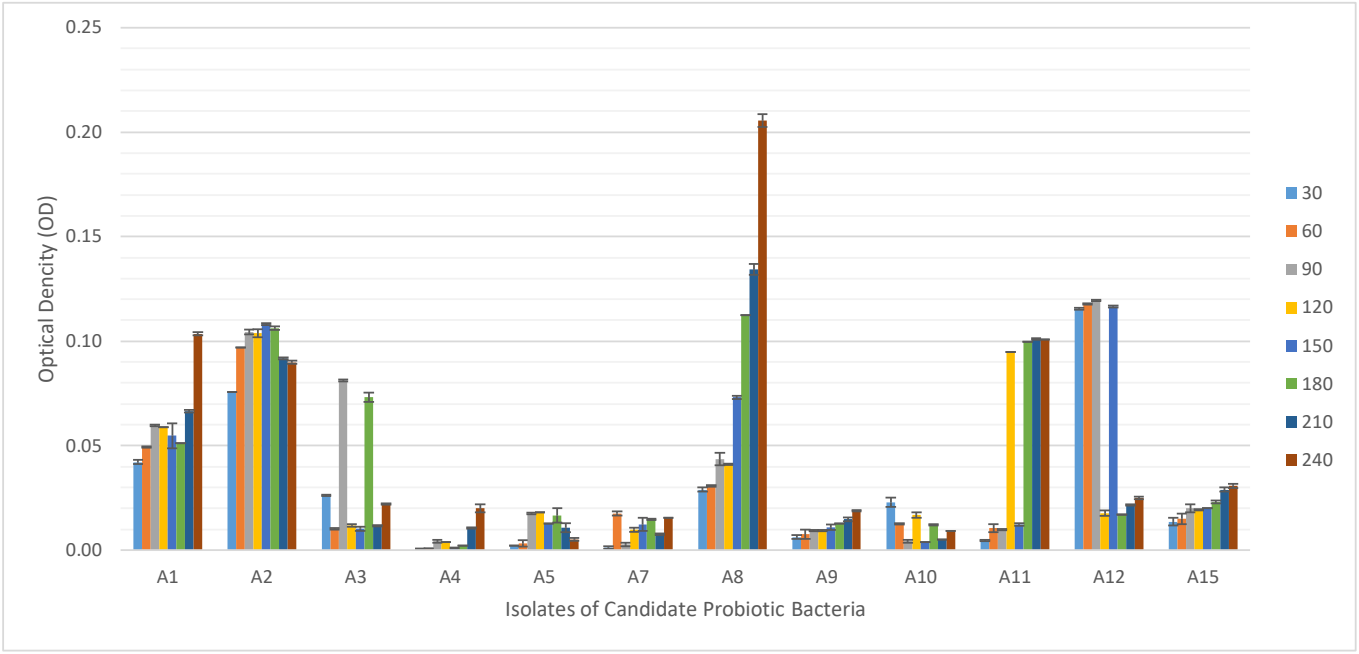

Fig 1. Growth of candidate isolates of probiotic bacteria that survival in 0,3% bile concentration. Measurement based on OD_{620} values from 30 until 240 minutes. Error bar represents SD.

Isolates with codes A11 and A12 showed pH 2 resistance (Fig.1), but were not able to grow well at 0.3% bile salt concentration (Fig.2). Isolate bacteria with code A8 were able to growth at 0.3% bile salt concentration but for 4 hours the growth of bacterial isolates decreased steadily, just as in pH 2 resistance (Fig.1). Generally, from ten isolates have potential as probiotic bacteria because they are able to pH 2 resistance and tolerant of 0.3% bile salt concentration even though the growth is different. Based on Figures 1 and 2, isolate with code A8 have the best potential for probiotic bacteria compared to other isolates. They are candidate of probiotic bacteria were taken to the next step of biochemical identification.

D. Biochemical Identification of Selected Candidate of Probiotic Bacterial

Isolates were observed as potential probiotic, next step is biochemical identification with used Vitex 2.0 compact cased type: ANC testing instrument 00001658F4A9 (12903). The results showed (Table 2) that three isolates were able to synthesize different carbohydrates and proteins. There is *Staphylococcus hominis* (A1), *Candida parapsilosis* (A2), *Anaerococcus prevotii* (A3), *Lactobacillus hilgardii* (A4), *Staphylococcus epidermidis* (A5-2), *Enterococcus faecalis* (A7, A9, and A15), *Candida tropicalis* (A8), *Lactobacillus plantarum* (A10), *Kocuria kristinae* (A11), and *Staphylococcus aureus* (A12).

Isolates A1, A2, and A11 are able to ferment compounds D-maltose and Alpha-Glucosidase. Isolate A2 has the ability to ferment more carbohydrate and protein compounds than the other two isolates. The result showed isolates A2 is type of microorganism that is able to grow rapidly in several media nutritional conditions.

Generally *Staphylococcus hominis* subsp. hominis is considered as non-pathogen [23]. *Staphylococcus hominis* MBBL 2–9 exhibited desirable probiotic traits, produced a bacteriocin with unique molecular weight and high antimicrobial activity similar to traditional antibiotics [21]. *S. hominis* strain MANF2 indigenous from Koozh (traditional fermented food product of South India) make it possible for development of new pharmaceuticals and functional food [24]. In the future, *S. hominis* will be probiotic bacteria that can be applied as functional food.

TABLE II
THE RESULT BIOCHEMICAL TESTS USING VITEX 2.0

Explanation	**Isolates**											
	A1	**A2**	**A3**	**A4**	**A5**	**A7**	**A8**	**A9**	**A10**	**A11**	**A12**	**A15**
Card type / Bar Code	GP / 24205421 03132022	YST / 24304831 03401262	ANC / 244047040 3501985	ANC / 24404704 03502197	GP / 24205421 03118990	GP / 24205421 03118983	YST / 243055520 3514553	GP / 24205421 03118983	ANC / 24404704 03501437	GP / 24250542 10342459 0	GP / 24205994 03323838	GP / 24205421 03118983
Ability to ferment	D-Maltose	D-maltose assimilation	D-Maltose	Arginine GP	Optochin Resistance	D-Maltose	D-Maltose Assimilation	D-Maltose	D-Mannose	D-mannose	D-Galactose	D-Maltose

Explanation	Isolates											
	A1	A2	A3	A4	A5	A7	A8	A9	A10	A11	A12	A15
	Alpha-Glucosidase	D-mannose assimilation	D-Mannose	Beta-Galactopyranosidase Indoxyl	O/129 resistance (comp. vibrio)	D-Mannose	D-Mannose Assimilation	D-Mannose	D-Cellobiose	Alpha-glucosidase	D-Trehalose	D-Mannose
	D-Trehalose	Arginine	D-Galactose	Leucin Arylamidase	Lactose	Arginine Dihydrolase	D-Galactose Assimilation	Arginine Dihydrolase	Saccharose / Sucrose	L-lactate alkalinization	Phosphatase	Arginine Dihydrolase
	Growth in 6,5% NaCl	D-galactose assimilation	Saccharose	Alpha-arabinose	Arginine Dihydrolase	Arginine Dihydrolase 2	D-Glucose Assimilation	Arginine Dihydrolase 2	Leucin Arylamidase	Growth in 6,5% NaCl	Bacitracin Resistance	Arginine Dihydrolase 2
	Optochin resistance	Alpha-Glucosidase	Maltotriose	5-bromo-4-chloro-3-indoxyl-alpha-galactoside	Urease	D-Galactose	D-Turanose Assimilation	D-Galactose	Arbutin	Leucin arylamidase		D-Galactose
	O/129 Resistance (comp.vibrio)	D-trehalose assimilation	Leucin Arylamidase	Beta-D-fucoside	Saccharose	D-Amygdalin	L-Glutamate Assimilation	D-Amygdalin	Esculin Hydrolysis	Alanine arylamidase		D-Amygdalin
	Urease	D-glucose assimilation	N-Acetyl-D-Glucosamine		Polymyxin B Resistance	Alanine Arylamidase	L-Proline Assimilation	Alanine Arylamidase	N-Acetyl-D-Glucosamine	Optochin resistance		Alanine Arylamidase
		D-turanose assimilation	d-Ribose 2		D-Maltose	D-Ribose	L-Malate Assimilation	D-Ribose	Phenylalanine Arylamidase	L-proline arylamidase		D-Ribose
		L-glutamate assimilation	D-Glucose		Phosphatase	Novobiocin Resistance	D-Trehalose Assimilation	Novobiocin Resistance	D-Glucose	Tyrosine arylamidase		Novobiocin Resistance
		L-proline assimilation	Phenylphosphonate		D-Galactose	Optochin Resistance	D-Xylose Assimilation	Optochin Resistance	5-bromo-4-chloro-3-indoxyl-beta-glucoside	Arginine dihydrolase		Optochin Resistance
		D-xylose assimilation	Urease		Bacitracin Resistance	Tyrosine Arylamidase	2-Keto-D-Gluconate Assimilation	Tyrosine Arylamidase	L-Pyrrolidonyl Arylamidase			Tyrosine Arylamidase
		N-acetyl-glucosamine assimilation	Argininse GP			O/129 resistance (comp. vibrio)	Leucin-Arylamidase Assimilation	O/129 resistance (comp. vibrio)				O/129 resistance (comp. vibrio)
		Saccharose/sucrose assimilation	L-Pyrrolidonyl Arylamidase			L-Aspartate Arylamidase	Methyl-A-D-Glucopyranoside Assimilation	L-Aspartate Arylamidase				L-Aspartate Arylamidase
		L-arabinose assimilation	Pyruvate			D-Sorbitol	D-Sorbitol Assimilation	D-Sorbitol				D-Sorbitol
		Acetate assimilation				Lactose	N-Acetyl-Glucosamine Assimilation	Lactose				Lactose
		D-gluconate assimilation				D-Mannitol	Gamma-Glutamyl-Transferase	D-Mannitol				D-Mannitol
		D-melezitose assimilation				Salicin	D-Melezitose Assimilation	Salicin				Salicin
		D-galacturonate assimilation				Urease	D-Galacturonate Assimilation	Urease				Urease

Explanation	Isolates											
	A1	**A2**	**A3**	**A4**	**A5**	**A7**	**A8**	**A9**	**A10**	**A11**	**A12**	**A15**
		2-keto-d-gluconate assimilation				N-Acetyl-D-Glucosamine	Citrate Assimilation	N-Acetyl-D-Glucosamine				N-Acetyl-D-Glucosamine
		Leucin-arylamidase				Saccharose	Alpha-Glucosidase	Saccharose				Saccharose
		Methyl-a-d-glucopyranoside assimilation				L-Pyrrolidonul-Arylamidase	Glucuronate Assimilation	L-Pyrrolidonul-Arylamidase				L-Pyrrolidonul-Arylamidase
		D-sorbitol assimilation				Polymycin B Resistance		Polymycin B Resistance				Polymycin B Resistance
		Citrate (sodium) assimilation				Metyl-B-D-Glucopyranosidase		Metyl-B-D-Glucopyranosidase				Metyl-B-D-Glucopyranosidase
		Glycerol assimilation				D-Trehalose		D-Trehalose				D-Trehalose
		Glucuronate assimilation				Alpha-Glucosidase		Alpha-Glucosidase				Alpha-Glucosidase
						Phosphatase		Phosphatase				Phosphatase
						Bacitracin Resistance		Bacitracin Resistance				Bacitracin Resistance
Result of identification	*Staphylococcus hominis*	*Candida parapsilosis*	*Anaerococcus prevotii*	*Lactobacillus hilgardii*	*Staphylococcus epidermidis*	*Enterococcus faecalis*	*Candida tropicalis*	*Enterococcus faecalis*	*Lactobacillus plantarum*	*Kocuria kristinae*	*Staphylococcus aureus*	*Enterococcus faecalis*

IV. Conclusion

Human origin and geographical location can directly affect the microbiota and fatty acid content in breast milk. In this study, probiotic bacteria from fifteen breast milk mother healthy from Jatinangor – Indonesia isolated are *Staphylococcus hominis* and *Lactobacillus plantarum*. *Staphylococcus hominis* has resistance pH 2 and tolerance of 0.3% bile concentration is best compared to other isolates. In the future, *S. hominis* will be probiotic bacteria that can be applied as functional food. Further research is needed on the determination of *Candida parapsilosis*, *Enterococcus faecalis*, *Candida tropicalis*, *Kocuria kristinae*, and *Staphylococcus aureus* because the five bacteria showed good resistance pH 2 and tolerance of 0.3% bile concentration.

Acknowledgment

We would like to thank Rector of Universitas Padjadjaran for the “Riset Fundamental Unpad” of Internal Grant from Universitas Padjadjaran. We also thanked to the Student Research Group, and Biofarma Microbiology Laboratory.

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