Pertanika J. Sci. & Technol. 24 (2): 351 - 370 (2016)

SCIENCE & TECHNOLOGY

Journal homepage: http://www.pertanika.upm.edu.my/

Identification, Characterisation and Phylogenetic Analysis of Commensal Bacteria Isolated from Human Breast Milk in Malaysia

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ABSTRACT

Human breast milk microbiota is essential for infant immune system development, maturation and protection against infection. However, there is scarce information on the fluid's microbiological composition from Malaysia. The objective of the study was to isolate, identify and characterise commensal bacterial population present in human breast milk from Malaysia. One hundred bacteria were isolated from the human breast milk of healthy lactating women (n=30). After preliminary screening, 20 isolates were characterised using both phenotypic and molecular techniques. The results indicated that most frequently identified bacteria in this study were *E. faecalis* and *S. hominis.* These organisms alongside *E. cloacae* were all metabolised D-Maltose, Sucrose, D-Turanose, α-D-Glucose, D-Fructose, D-Mannose, D-Galactose, D-sorbitol and D-Mannitol and were able to grow at pH 5 and 6, 1% sodium lactate, 1%, 2% and 8% NaCl. BLAST showed over 99% similarity to those deposited in Genbank. Phylogenetic-relatedness was depicted using neighbour-joining method and had two clades with 100% bootstrap. These findings provided insight into the nature, characteristics and also phylogenetic-relatedness of bacteria present in human milk from Malaysia. Isolation and identification of commensal bacteria from human milk are considered the first step for future studies on the benefit of these organisms towards human health.

Keywords: Commensal bacteria, human breast milk, Malaysia, phenotype, phylogeny

Article history:
Received: 25 July 2015
Accepted: 28 January 2016

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ISSN: 0128-7680

INTRODUCTION

Human breast milk is a vital postpartum fluid and is considered the most important element in metabolic and immunologic programming of the health of neonates (Wagner, 2012). The presence of live cells in the fluid has been established across the world but such information from Malaysia is missing. Microbial populations in human milk vary with geographical location (Dubos et al., 2011; Nasiraii et al., 2011; Nuraida et al., 2012). Thus, the aim of this study was to isolate, identify and characterise bacteria present in human milk from Malaysia.

Human milk is an important factor in the initiation, development and of course, the composition of the neonatal gut microflora as it is a source of microorganisms to the infant gut for several weeks after birth (Martin et al., 2005). Wagner (2012) defined breast milk as a bioactive fluid that evolved from colostrum to mature milk. Heikkilä and Saris (2003) and Martín et al. (2002) estimated the ingestion of about 1×10^5 to 1×10^7commensal bacteria by infants consuming approximately 800 mL per day while suckling.

The human body is inhabited by 10 times more bacteria than body cells. Reports evaluating the beneficial effects of these bacteria have been shared in past decades (Gregoret et al., 2013). Human breast milk is not an exception as it contains numerous live cells. It is considered a symbiotic fluid. It is not sterile, as traditionally believed. Human breast milk is a complex fluid rich in nutritional and non-nutritional bioactive components (Prentice, 1996). These include prebiotic oligosaccharides (Kralj et al., 2002; Pridmore et al., 2004; Nasiraii et al., 2011) in addition to the potential probiotic and the biotherapeutic commensal (Holzapfel et al., 1998; Martin et al., 2005; Nasiraii et al., 2011).

Research on commensal bacteria from human milk has just resumed as previously breast milk studies focused on pathogens (Heikkila & Saris, 2003). Commensal bacterial strains reported from human milk are widely used in the health and industrial sectors as they have the ability to inhibit the growth of a wide spectrum of pathogenic bacteria by competitive exclusion and/or through the production of antimicrobial compounds, such as bacteriocins, organic acids and hydrogen peroxide (Fooks et al., 1999; Reid, 2001; Beasley, 2004; Martín et al., 2007). Martin et al. (2005) and Olivares et. al. (2006) suggested that the milk of healthy woman may be a source of potentially probiotic or biotherapeutic bacteria and can play a role in protecting mothers and/or infants against infectious and clinical diseases. In the present study, we characterised the commensal bacterial composition of expressed breast milk in Malaysia and determined their evolutionary relationship by sequence and phylogenetic analyses.

MATERIALS AND METHODS

Sample Collection

Expressed breast milk was collected from 30 healthy women aged between 23 and 38 years who were breastfeeding children from birth to two years old. They were randomly selected from four states and two federal territories in Peninsular Malaysia, namely Selangor, Johor, Perak, Negeri Sembilan, Kuala Lumpur and Putrajaya. Consent forms were obtained from the respondents. The samples were received as freshly expressed, chilled or iced. All samples collected were immediately stored at -20 ºC until further analyses.

Bacterial Culture and Isolation

A total of 100 isolates were obtained by plating 100 µL of the milk sample on non-selective MRS agar plates after thawing at 4 ºC overnight. The plates were incubated aerobically at 37 ºC for 48 to 72 h. An MRS medium was used to support growth of nutritionally fastidious microbes as previously reported (Heikkila & Saris, 2003). Based on colony morphology, colonies were randomly picked from culture plates and purified twice in the same conditions for 18 to 24 h. The purified strains were stored at -80ºC in growth media containing 20% (v/v) glycerol.

Bacterial Identification and Phenotyping

Isolates were identified and metabolically profiled using Biolog GenIII MicroPlate™ microbial identification system (Biolog; Haywood, CA, USA). Pure bacterial isolates were suspended in minimal media (Inoculating Fluid A (IF-A); Biolog; Haywood, CA, USA) using a sterile cotton swab and adjusted to approximately 90 to 98% transmission using a Biolog turbidimeter. From the bacterial suspension, 100µL was inoculated into each well of GenIII micro titre plates. The plates were incubated at 33 ºC for 16 to 22 h. Bacteria were speciated using algorithms provided by ML5 on the Biolog microstation 2 reader (Microlog 3/5.2.01 35, Biolog, USA). For all isolates, end-point raw absorbance data from the 96-well GenIII identification plates were normalised by subtracting absorbance in the negative control well and then exported automatically into a separate programme (Statistica†; Statsoft, Tulsa, OK, USA) for data analysis. Isolate metabolic profile was compared to the Biolog identification database (Biolog GEN III 2_6_1_08.15G). All isolates identified as microaerophilic based on the results obtained using IF-A were double confirmed with group specific fluid - IF-C (Biolog; Haywood, CA, USA).

Molecular Identification and Typing of the Isolates

Bacterial genomic DNA was extracted using the Qiagen DNA extraction kit (QIAamp®, Hilden, Germany) according to the protocols of Gram positive bacteria from DNeasy Blood and tissue culture handbook (2006) with modifications. Prior to the addition of buffer AL, 10µL of RNase A/T1 (2mg/mL, Fermentas) was added to the mixture after Proteinase K, vortexed and incubated at room temperature for 2 min. The genomic DNA was eluted with 50µL of sterile deionised distilled water instead of 200 µL of elution buffer and then stored at -20°C.

The extracted bacterial genomic DNA was visualised using 1% Agarose (TopVision™, Thermo scientific, Fermentas) stained with 1µL of Redsafe™ (iNtRoN Biotechnology, Korea) at 99V for 30 min. UV illuminator (G-Box, Syngene) with Gene system software (version 1.4.0.0) was used to view and capture the gel image.

Amplification was carried out using a Bio-Rad Thermocycler (Berkeley, California). The reaction mixture (25µL) contained 12.5µL of PCR master mix (DreamTaq Green, Thermo scientific, USA), 10.5µL of nuclease free water, 1µL of genomic DNA template and 0.5µL of 10mM of forward and reverse primers; 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and WLAB2R (5′-TCGAATTAAACCACATGCTCCA-3′) (Integrated DNA Technologies) as previously described by Emerenini et al. (2013) and Lopez et al. (2003). The amplification

programme was initial denaturation at 95 ºC for 2 min, followed by 30 cycles of denaturation at 95 ºC for 0.5 min, primer annealing at 48.9 ºC for 0.5 min and extension at 72 ºC for 1.5 min. Final extension was done at 72 ºC for 7 min.

The PCR products were purified using the GelJet PCR purification kit (Thermo-scientific, Lithuania) according to the manufacturer's instructions. The purified PCR amplicon were visualised on 1% agarose gel as described previously. The DNA concentrations of the purified products were determined using a nanodrop (IMPLEN, Shimadzu) before subjected to sequencing.

DNA Sequencing and Sequence Analysis

The purified PCR products of each isolate were sequenced thrice on both strands by ABI Prism 3730xl DNA sequencer using ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer) to get precise consensus sequence. BioEdit™ software version 7.2 package (Hall, 1999) was used in editing, quality assurance trimming and analysing the consensus sequence between the three sets of forward and the reverse complement of the reverse sequences. The consensus sequence was then BLAST for highly similar organism sequence identity using the "blastn" algorithm from GenBank (http://www.ncbi.nlm.nih.gov/BLAST/). Isolate identities of 99% and above were considered the identity of the query organisms. Sequences of all isolates from each genus were BLAST with a reference sequence for comparison.

Molecular Evolutionary Genetics Analysis, MEGA (Tamura et al., 2007) software version 6.03 Beta (6131108) was used to construct the tree. The neighbour-joining algorithm was used to explore the phylogenetic tree (Saitou & Nei, 1987); the robustness of individual branches was estimated using bootstrapping with 1000 replications (Felsenstein, 1985).

A total of 35 isolates was included in the phylogenetic analysis depicted by neighbour-joining (Table 1). Twenty of the sequences were obtained from this study while 15 closely related available sequences were retrieved online from the GenBank database.

Table1
16S rDNA Gene of the Strains Used in Sequence and Phylogenetic Analyses

S/N	Strain	Accession number	Region	References
1	*Enterococcus faecalis*	CP008816	1629243-1630110	Minogue et al. (2014)
2	*Enterococcus faecalis*	CP008816	2056343-2057210	Minogue et al. (2014)
3	*Staphylococcus lugdunensis*	FR87027	2424570-2425475	Heilbronner et al. (2011)
4	*Staphylococcus lugdunensis*	FR87027	2476052-2476957	Heilbronner et al. (2011)
5	*Staphylococcus lugdunensis*	CP001837	2447081-2447986	Tse et al. (2010)
6	*Staphylococcus lugdunensis*	CP001837	2505017-2505922	Tse et al. (2010)
7	*Staphylococcus hominis*	FN393804	10-900	Unpublished
8	*Staphylococcus hominis*	EU337115	24-914	Bauer et al. (2009)
9	*Staphylococcus hominis*	FJ768458	15-905	Tothova et al. (2010)
10	*Staphylococcus hominis*	JQ795892	5-895	Unpublished

Table1 (*continue*)

11	*Staphylococcus hominis*	KJ147074	14-904	Unpublished
12	*Enterobacter cloacae*	CP008823	3604651-3605545	Unpublished
13	*Enterobacter cloacae*	CP008823	4730135-4731029	Unpublished
14	*Enterobacter cloacae*	CP008823	4390718-4391612	Unpublished
15	*Enterobacter cloacae*	CP008823	4649858-4650752	Unpublished
16	*Staphylococcus hominis*	KM392086	1-890	This study
17	*Staphylococcus hominis*	KM392085	1-890	This study
18	*Staphylococcus lugdunensis*	KM392088	1-905	This study
19	*Staphylococcus hominis*	KM392087	1-890	This study
20	*Staphylococcus hominis*	KM609184	1-890	This study
21	*Staphylococcus hominis*	KM609185	1-890	This study
22	*Enterobacter cloacae*	KM609192	1-894	This study
23	*Enterobacter cloacae*	KM392095	1-894	This study
24	*Enterobacter cloacae*	KM392093	1-894	This study
25	*Enterobacter cloacae*	KM392094	1-894	This study
26	*Enterococcus faecalis*	KM609186	1-867	This study
27	*Enterococcus faecalis*	KM392090	1-867	This study
28	*Enterococcus faecalis*	KM392089	1-867	This study
29	*Enterococcus faecalis*	KM392091	1-867	This study
30	*Enterococcus faecalis*	KM609187	1-867	This study
31	*Enterococcus faecalis*	KM609188	1-867	This study
32	*Enterococcus faecalis*	KM609189	1-867	This study
33	*Enterococcus faecalis*	KM609190	1-867	This study
34	*Enterococcus faecalis*	KM609191	1-867	This study
35	*Enterococcus faecalis*	KM392092	1-867	This study

Confirmatory PCR

After the molecular identification of the isolates, confirmatory PCR was performed using genus and/or specie specific primers (Table 2) to the identified isolates to double confirm the success and reliability of the bacterial identification.

Bacterial Accession Numbers

All sequences obtained in this study were deposited in the GenBank under the following accession numbers: KM392085, KM392086, KM392087, KM392088, KM392089, KM392090, KM392091, KM392092, KM392093, KM392094, KM392095, KM609184, KM609185, KM609186, KM609187, KM609188, KM609189, KM609190, KM609191 and KM609192.

Table 2
Primers Used for Confirmatory PCR

S/N	Name	Sequence (5′ 3′)	PCR product (bp)	Remark	Reference
1	E1	TCA ACC GGG GAG GGT	733	*Enterococci* genus specific primer	(Deasyt et al., 2000)
	E2	ATT ACT AGC GAT TCC GG			
2	FL1	ACTTATGTGACTAACTTAACC	360	*Enterococcus faecalis* species specific primer	(Jackson et al., 2004)
	FL2	TAATGGTGAATCTTGGTTTGG			
3	TstaG422 F	GGCCGTGTTGAACGTGGTCAAATCA	370	Staphylococcus genus specific primer	(Martineau et al., 2001; Morot-bizot et al., 2004)
	Tstag765 R	TNACCATTTCAGTACCTTCTGGTAA			
4	Hsp60-F	GGTAGAAGAAGGCGTGGTTGC	341	*Enterobacter cloacae* species hsp60 gene specific primer	(Morand et al., 2009)
	Hsp60-R	ATGCATTCGGTGGTGATCATCAG			

RESULTS AND DISCUSSION

Human milk was traditionally considered sterile; however, studies have shown that the fluid serves as a continuous supply of commensal, mutualistic and/or potentially probiotic bacteria to the infant gut. Staphylococci, Streptococci, Lactic acid bacteria and Bifidobacteria were reported to be the dominant species in human milk (Fernández et al., 2013).

Twenty bacterial isolates were identified in this study and their metabolic profile showed that all of the isolates were able to metabolise a wide variety of carbon sources and have the ability to survive in many environments. For example, D-Maltose, Sucrose, D-Turanose, α-D-Glucose and D-Fructose were metabolised by all of the bacteria identified. They vary in a wide range of growth requirements, including also the carbon sources they utilise. Most of the identified bacteria needed some additional growth factors for adequate growth. Pyruvate, alanine, arginine, aspartate, glutamate, histidine, serine and pectin are some examples of essential nutrients required by these bacteria, which may not be present in general growth media. Therefore, we used an enriched media for their growth; otherwise they can over-grow in mixed cultures. Probiotic characteristics including acid tolerance and *l*-lactate production as seen from the isolates were also observed as did Choi et al. (2006). Individual requirement differences are tabulated in Table 3.

Consistent with earlier studies, this study showed the genera *Enterococci* and *Staphylococci* were the most frequently isolated from human breast milk (Caroll et al., 1979; Eidelman & Szilagyi, 1979; El-Mohandes et al., 1993; Wright & Feeny, 1998; Heikkila & Saris, 2003). Among the 20

Table 3
Metabolic Profiles of the Bacteria Isolated

Carbon sources	*Staphylococcus hominis* (GP cocci)	*Enterococcus faecalis* (GP cocci)	*Enterobacter cloacae ssdissolvens* (GN ENT)
Dextrin	-	+	+
D-Maltose	+	+	+
D-Trehalose	+	+	+
D-cellobiose	-	+	+
Gentibiose	-	+	+
Sucrose	+	+	+
D-Turanose	+	+	+
D-Stachyose	-	-	+
D-Raffinose	-	-	+
α-D-Lactose	-	-	+
D-Melibiose	-	-	+
β-Methyl-D-Glucosidase	-	+	+
D-salicin	-	+	+
N-Acetyl-D-Glucosamine	+	-	+
N-Acetyl-β-D-Mannosamine	-	+	+
N-Acetyl-D-Galactosamine	-	+	+
N-Acetyl Neuraminic Acid	+	-	-
α-D-Glucose	+	+	+
D-Mannose	-	+	+
D-Fructose	+	+	+
D-Galactose	-	+	+
L-Rhamnose	-	-	+
Inosine	-	+	+
D-sorbitol	-	+	+
D-Mannitol	-	+	+
Myo-innositol	-	-	+
Glycerol	-	+	+
D-Glucose-6-PO_4	-	+	+
D-Fructose-6-PO_4	-	+	+
D-Serine	-	-	+
Glycyl-L-Proline	-	-	+
L-Alanine	-	-	+
L-Arginine	-	-	+
L-Aspartic Acid	-	-	+
L-Glutamic Acid	-	-	+
L-Histidine	-	-	+
L-Serine	+	+	+
Pectin	+	+	+

Table 3 (*continue*)

Carbon sources	*Staphylococcus hominis* (GP cocci)	*Enterococcus faecalis* (GP cocci)	*Enterobacter cloacae ssdissolvens* (GN ENT)
D-Galactoronic Acid	-	-	+
L-Galactoronic Acid Lactone	-	-	+
D-Gluconic Acid	-	-	+
D-Glucoronic Acid	-	+	+
Glucoronamide	-	-	+
Mucic Acid	-	-	+
D-Sacchartic Acid	-	-	+
D-Hydroxy-Phenylacetic Acid	-	-	+
Methyl Pyruvate	+	+	+
L-Lactic Acid	+	-	+
Citric Acid	-	+	+
L-Malic Acid	-	+	
Bromo-Succinic Acid	-	+	-
α-Keto-Butytic Acid	-	+	-
ϒ-Amino-Butryric Acid	-	-	-
Acetic acid	+	-	+
Formic Acid	-	-	+

+ = Ability to metabolise - =Unable to metabolise GP=Gram Positive

bacteria isolated, *E. faecalis* were 10 and *Staphylococci*, six. The genus *Staphylococcus* has species that are of concern in medical treatment; however, most of the detected *Staphylococci* (*S. hominis*) are known as residents of the normal bacterial flora in humans (Heikkila & Saris, 2003).

This present finding supports the view that commensal *Staphylococci* are part of the predominant bacterial species in breast milk (Heikkilä & Saris, 2003). This bacterium may have originated from the skin (Dubos et al., 2011), perhaps when it came into contact with milk during suckling or pumping. Some bacteria present in the maternal gut could reach the mammary gland during late pregnancy and lactation through a mechanism involving gut monocytes. Infrared photography has shown that a certain degree of retrograde flow back into the mammary ducts can occur during suckling (Fernández et al., 2013). This bacterial community may differ depending on the individual and the health status of the lactating women. Cabrera-Rubio et al. (2012) held the view that breast milk microbiome is by the mammary glands because the bacteria isolated during the weeks previous to labour, that is, before any kind of contact with the infant, were very similar to the bacterial species isolated from fresh milk obtained after childbirth.

E. faecalis is a common inhabitant of the gastrointestinal tracts of humans and is a highly diverse species that includes opportunistic pathogens, commensal, food-derived and probiotic strains. However, probiotic strains were genetically similar to those from diary products

(Buhnik-Rosenblau et al., 2013). It is a member of the lactic acid bacteria group and was found as the most frequently identified strain in this study (Table 5). Its origin in the milk remains unclear. Some reports suggest that these microorganisms and even Bifidobacteria might be brought to the lactating breast tissue through the endogenous trafficking of bacteria-loaded dendritic cells originated from intestinal mucosa (Dubos et al., 2011). Dendritic cells have been described to penetrate the intestinal epithelium to take up commensal bacteria from the gut lumen to reach the systemic circulation, and to retain even life bacteria for several days (Cabrera-Rubio et al., 2012). However, it has been reported that lactobacilli and enterococcal isolates present in human milk are genotypically different from those isolated in the skin, within a same bacterial species and a same host (Martín et al., 2003).

A chemical sensitivity assay obtained in this study showed that all of the isolates profiled were able to grow in harsh environments such as growth at low acidic conditions (pH 5 and 6), as well as in a salted environment: that is in the presence of 1% sodium lactate and 1%, 2% and 8% NaCl. Thus, these characteristics make them potential probiotic microorganisms. These characteristics can be compared with probiotics' ability to resist bile acid and stomach conditions. The isolates vary in resistance to common antibiotics. For example, *Enterobacter cloacae* isolates were found to be resistant to Lincomycin, Niaproof 4, Rifamycin SV and Vancomycin antibiotics while *Staphylococcus hominis* and *Enterococcus faecalis* are sensitive. The latter, which were reported to be commensals/probiotic, were found to be sensitive to a range of antibiotics; nonetheless they survived in unfavourable physical environments (Table 4).

Table 4
Chemical Sensitivity Assay of the Isolated Bacteria

Chemicals	*Staphylococcus hominis* (GP cocci)	*Enterococcus faecalis* (GP cocci)	*Enterobacter cloacae ssdissolvens* (GN ENT)
pH 6	+	+	+
pH 5	+	-	+
1% NaCl	+	+	+
2% NaCl	+	+	+
8% NaCl	+	+	-
1% Sodium lactate	+	+	+
Fusidic Acid	-	-	+
D-Serine	+	+	-
Troleandomycin	-	-	+
Rifamycin SV	-	-	+
Minocycline	-	-	-
Lincomycin	-	-	+
Guanidine HCl	-	+	+
Niaproof 4	-	-	+
Vancomycin	-	-	+
Tetrazollium violet	+	+	+
Tetrazollium blue	-	+	+

Table 4 (*continue*)

Chemicals	*Staphylococcus hominis* (GP cocci)	*Enterococcus faecalis* (GP cocci)	*Enterobacter cloacae ssdissolvens* (GN ENT)
Nalidixic Acid	+	+	-
Lithium Chloride	+	+	-
Potassium Telluite	+	+	-
Aztreonam	+	+	-
Sodium Butyrate	+	+	-

+ = Resistant - = Sensitive

Table 5
Bacterial Species Recovered from Breast Milk Samples

S/N	Sample no.	Incubation time (days)	Shapes of organism	Gram reaction	Bacterial species
1	1	2	Cocci	+ve	
2	2	4			
3	2a1		Cocci	+ve	*Enterococcus faecalis* KM609186
4	3	4			
5	3a1		Rods	+ve	
6	3a2		Cocci	+ve	*Staphylococcus hominis* KM609184
7	3b		Cocci	+ve	*Staphylococcus hominis* KM392086
8	4	3	Cocci	+ve	
9	5	4			
10	5a1		Cocci	+ve	*Enterococcus faecalis* KM392090
11	6	6	No growth		
12	7	6	No growth		
13	8	3			
14	8a2		Cocci	+ve	*Enterococcus faecalis* KM392089
15	9		Cocci	+ve	
16	10	3			
17	10a		Cocci	+ve	*Staphylococcus hominis* KM392085
18	10c		Cocci	+ve	*Staphylococcus hominis* KM609185
19	11	3	Rods	+ve	
20	12	4	Cocci	+ve	
21	13	3	Cocci	+ve	

Table 5 (*continue*)

S/N	Sample no.	Incubation time (days)	Shapes of organism	Gram reaction	Bacterial species
22	14	3	Cocci	+ve	
23	15	3	Cocci	+ve	
24	16	3	Rods	+ve	
25	17	4	Cocci	+ve	
26	18	4			
27	18g		Cocci	+ve	*Enterococcus faecalis* KM392091
28	18n		Cocci	+ve	*Staphylococcus lugdunensis* KM392088
29	19	4			
30	19b1		Cocci	+ve	*Enterococcus faecalis* KM609187
31	19b2		Cocci	+ve	*Staphylococcus hominis* KM392087
32	20	4	Cocci	+ve	
33	21	3	Cocci	+ve	
34	22	2			
35	22l		Cocci	+ve	*Enterococcus faecalis* KM609188
36	22n		Rods	-ve	*Enterobacter cloacae* KM392094
37	23	3	Cocci	+ve	
38	24	4			
39	24d		Rods	-ve	*Enterobacter sp.* KM609192
40	24i		Rods	-ve	*Enterobacter sp.* KM392095
41	24m		Rods	-ve	*Enterobacter sp.* KM392093
42	25	4			
43	25a1		Cocci	+ve	*Enterococcus faecalis* KM609189
44	26	5	Cocci	+ve	
45	27	2			
46	27b1		Cocci	+ve	*Enterococcus faecalis* KM609190
47	28	4			
48	29	2			
49	29a1		Cocci	+ve	*Enterococcus faecalis* KM609191
50	30	3			
51	30a2		Cocci	+ve	*Enterococcus faecalis* KM392092

Resistance to bile salts, tolerance to gastric acid and low pH conditions are indication of survival in the stomach and intestinal digestion indicating probiotic properties (Dunne et al., 2001). As reported previously (Nueno-Palop & Narbad, 2011), *E. faecalis* is one of the strains found in this study to possess these characteristics. It was the strain that showed the most adherence to human intestinal cells (Nueno-Palop & Narbad, 2011) and adherence is an effective property of potential biotherapeutics as it is a prerequisite for colonisation, making the organisms a good probiotic.

S. hominis is normally found on human skin and usually as a harmless commensal. It is a coagulase-negative member of the bacterial genus *Staphylococcus*. They are Gram-positive, spherical cells in clusters of usually 1-2 mm in diameter. On agar plates, colonies usually have wide edges and an elevated centre and are yellow-orange pigmented. As observed during our isolation and also reported in previous findings (Abdalla et al., 2013), this study found out that *S. hominis* was able to metabolise glucose, fructose, sucrose, trehalose, turanose, lactose, galactose, mannitol and mannose. Its optimal growth conditions were 28 to 40 °C, but good growth was still observed at 45 °C, NaCl around 7.5% (Abdalla et al., 2013). *S. hominis* differ phenotypically from other species in the *Staphylococcus* genus as it produces acid from trehalose, low tolerance of NaCl and the carbohydrate reaction pattern.

The issue of antibiotic resistance is of medical concern since most of the genes involved are often plasmid or transposon associated, thereby presenting a risk of horizontal gene transfer of such genes to pathogens. In line with Nueno-Palop & Narbad (2011), the present findings indicated that both *E. faecalis* and *S. hominis* were susceptible to common antibiotics, for example, vancomycin, Niaproof 4 and rifampicin. However, *E. cloacae* is of concern due to its antibiotic-resistant pattern.

Pathogens such as *Staphylococcus aureus* and *Streptococcus agalactiae*, have been reported in breast milk (Bingen et al., 1992; Le Thomas et al., 2001; Novak et al., 2000). However, such pathogens were regarded as rare contaminants of the breast milk of healthy lactating women (Caroll et al., 1979; West et al., 1979; Law et al., 1989; El-Mohandes et al., 1993). In this study, the isolation of *Enterobacter cloacae* from the milk of healthy women can be considered as a contamination of breast milk. The presence of maternal dietary fat during pregnancy and lactation greatly influences breast milk fatty acid content (Amusquivar et al., 2000; Scopesi et al., 2001). Maternal dietary and health conditions (especially in terms of infection) are very important to the nature of bacteria in the milk. Berlin et al. (1984) reported the presence of dietary caffeine in human milk shortly after its consumption.

The number of *E. cloacae* in this study was few, but if the numbers were very high in the breast milk it could have caused dissemination of multidrug resistance. This is because the isolate was able to resist the presence of common antibiotics such as rifamycin SV, lincomycin, vancomycin, troleandomycin, among others while also being able to metabolise a wide range of carbon sources, growth at low pH and withstand up to 8% NaCl similar to *E. faecalis* and *S. hominis*.

Biolog GenIII MicroPlate™ was developed originally for the identification of isolated microorganisms based on their substrate utilisation pattern. It is a 96-well microtiter plate containing tetrazolium violet with a 71-carbon source utilisation assay and 23 chemical

sensitivity assays in addition to positive and negative controls. Three species of bacteria (*E. faecalis*, *E. cloacae*, *S. hominis*) were phenotyped using this identification panel.

Human milk is generally accepted as the best source of nutrition for neonates. It contains all of the essential nutrients and growth factors that can protect infants from infections. Expressed human milk contains commensal bacteria (Heikkilä & Saris, 2003) and has the potential to replace fresh milk. In addition to nutritional support, breast milk provides bioactive constituents that both directly and indirectly enhance mucosal barrier function and shape immune development (Cabrera-Rubio et al., 2012). The species diversity and the importance of the normal bacterial flora have received little attention so far (Heikkilä & Saris, 2003). This study explored the commensals' potential probiotic bacterial diversity in expressed human milk of healthy lactating women in Peninsular Malaysia.

Human breast milk is a fluid that serves as a source of microbes and their growth factors. It regulates interactions of host-microbe (Cabrera-Rubio et al., 2012). Until the past decade, most reports on the bacteria in human milk have focused on pathogenic bacteria (Gregoret et al., 2013), probably due to its significance in breast milk for milk banks. However, the milk contains beneficial bacteria whose growth is supported by several factors such as lysozyme, lactoferrin and oligosaccharides (prebiotics), which is believed to have assisted in preventing infections (Cabrera-Rubio et al., 2012).

All genomic DNA gel bands viewed have greater or equal to 20,000 base pairs (bp) when compared to the bands of the DNA ladder (Thermo Scientific). PCR amplification using the 16S rDNA gene universal primer set generated amplicons of around 945bp in size (Figure 1A). This is in line with the results of a previous study (Emerenini et al., 2013). BLAST results of the sequences obtained in this study showed an identity query coverage percentage of 100% and the percentage identity was 99% and 100%.

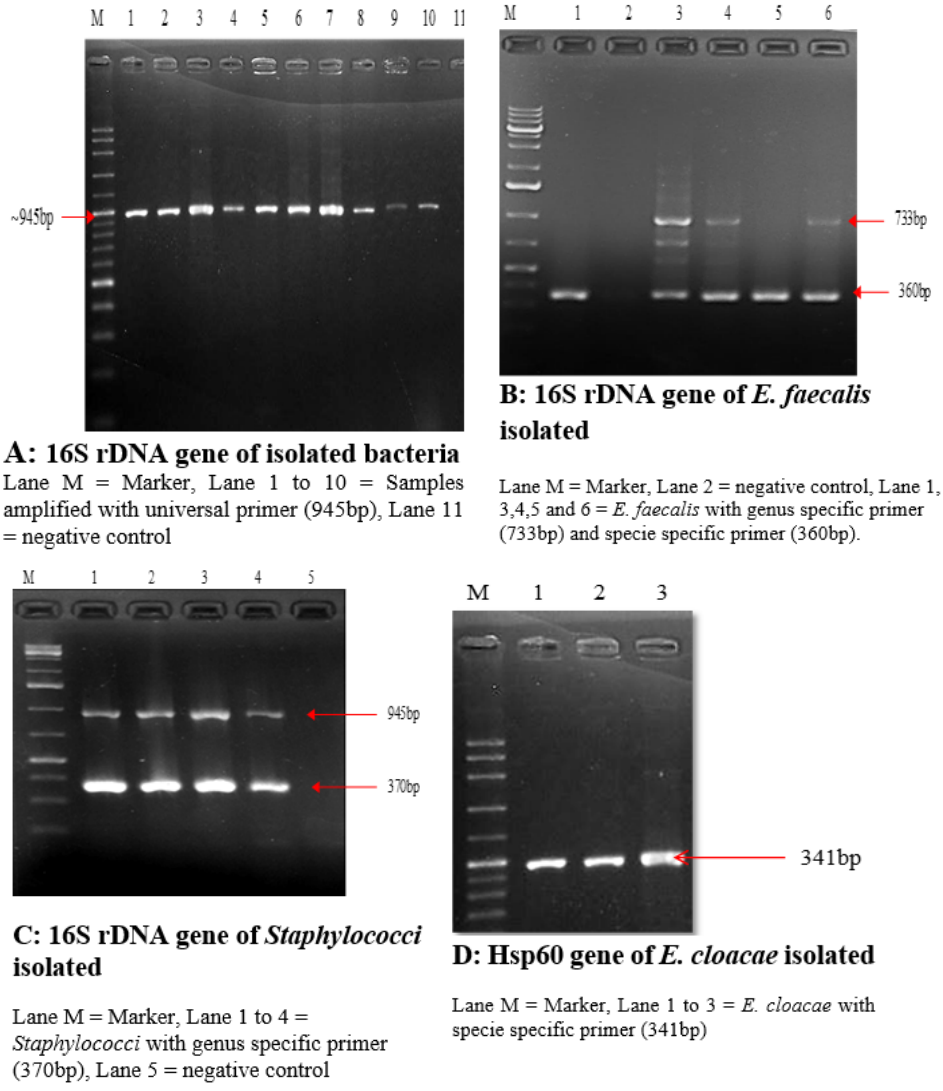
The *Enterococcus faecalis* genus- and species-specific primer sets produced fragments of around 733bp and 360bp, respectively (Figure 1B), as reported by Deasyt et al. (2000) and Jackson et al. (2004). Efforts to get clear and sharper bands for both genus- and species-specific primers for *E. faecalis* proved abortive, with PCR programmes annealing at different temperatures ranging from 47^{o}C to 62^{o}C. All the isolates yielded the expected band sizes with specie-specific primers and so, all were considered *E. faecalis*.

The *Staphylococci* group identified by sequencing and phenotyping produced bands with genus-specific primers at an annealing temperature of 49^{o}C. Products of 370 bp were produced by confirmatory PCR carried out using the *Staphylococci* genus primer set (Figure 1C), which was in line with that reported by Martineau et al. (2001).

Three out of four isolates produced bands when the *Enterobacter* species was amplified using the hsp60 gene of *Enterobacter cloacae*, as reported by Morand et al. (2009) and produced approximately 341bp (Figure 1D). However, the one *Enterobacter* isolate that failed to produce a band may be of a different species.

All sequences obtained were grouped by their respective genera and blasted against a reference. *E. faecalis* group was blasted using *Enterococcus faecalis* ATCC 29212, with the complete genome isolated in the USA with Sequence ID: gb|CP008816.1 and Length: 2939973 base pairs. Our isolates were found to amplify position 1629207 to 1630144 of the reference

sequence. Multiple sequence alignment was performed by the ClustalW programme of the BioEdit™ software package version 7.2. The sequences were trimmed at position 1629243-1630110 of the reference sequence so that they all had the same number of bases. Therefore, a total of 867 base pairs was used in *Enterococcus faecalis* multiple sequence alignment (data not shown).



A: 16S rDNA gene of isolated bacteria

Lane M = Marker, Lane 1 to 10 = Samples amplified with universal primer (945bp), Lane 11 = negative control

B: 16S rDNA gene of *E. faecalis* isolated

Lane M = Marker, Lane 2 = negative control, Lane 1, 3,4,5 and 6 = *E. faecalis* with genus specific primer (733bp) and specie specific primer (360bp).

C: 16S rDNA gene of *Staphylococci* isolated

Lane M = Marker, Lane 1 to 4 = *Staphylococci* with genus specific primer (370bp), Lane 5 = negative control

D: Hsp60 gene of *E. cloacae* isolated

Lane M = Marker, Lane 1 to 3 = *E. cloacae* with specie specific primer (341bp)

Figure 1. PCR amplification of the isolated bacteria from human breast milk.

The *Enterobacter* group was blasted using *Enterobacter cloacae* ECNIH2 as the reference genome, the complete genome of which was also isolated in the USA with Sequence ID: gi|662712225, accession number CP008823 and length of 4852980 base pairs. Our isolates were found to amplify position 3604643 to 3605545 of the reference sequence. Multiple sequence alignment was performed by the ClustalW programme of the BioEdit™ software

package version 7.2. The sequences were trimmed at position 3604651 to 3605545 of the reference sequence so that they all had the same number of bases. Therefore, a total of 894 base pairs were used in the *Enterobacter* group for multiple sequence alignment (data not shown).

In the *Staphylococcus* genus, there were two strains: *S. lugdunensis* and *S. hominis*. *S. lugdunensis* was blasted against the complete genome of *Staphylococcus lugdunensis* N920143 with the sequence ID: gi|339893212, accession number FR870271 and length of 2595888 base pairs. Our isolate matched the reference at position 2424570 to 2425475. All of the sequences aligned at position 2424570 to 2425475 of *S. lugdunensis* (FR870271). The multiple sequence alignment included a total of 905 bases (data not shown).

Due to the lack of an available complete genome sequence of *Staphylococcus hominis*, the group was blasted against the *Staphylococcus* group (taxid: 90964). Our sequences were found to match 100% to *S. hominis* partial 16S ribosomal RNA with accession number KJ147074, which was 1511bp in length and had a sequence ID of gi|628823726 at region 4 to 944 base pairs. Multiple sequence alignment matched *S. hominis* (KJ147074) at 14 to 904; that is, a total of 890 base pairs were included in the alignments (data not shown).

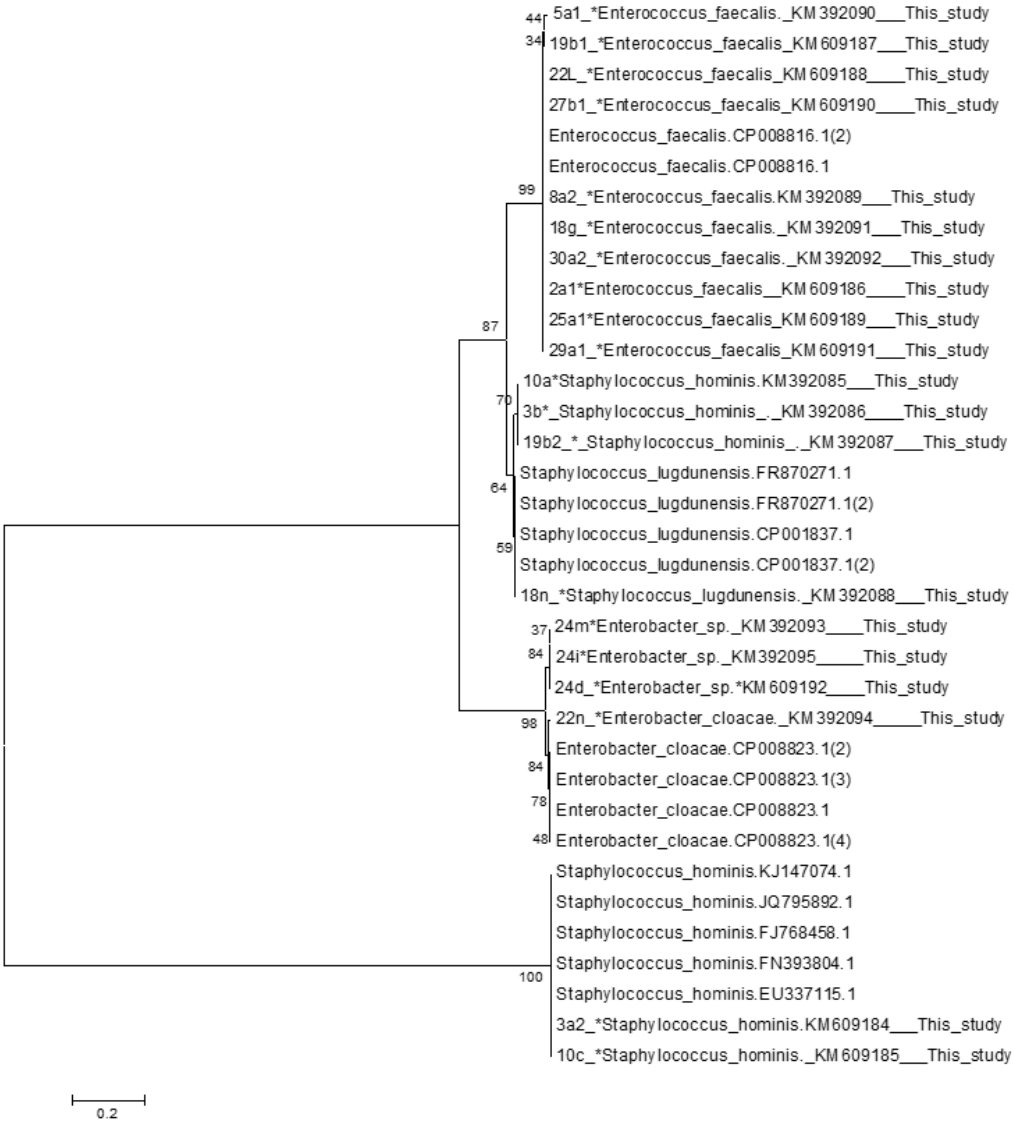
The phylogenetic tree constructed (Figure 2) had two main clades, each containing an isolate relating closely to other isolates in the same genus. However, the *Staphylococcus* genus was divided into two distant clades. Each clade had a high bootstrap value (>80%). One of the clades contained *S. hominis* and *S. lugdunensis* from our isolates and the *S. lugdunensis* reference sequences, while the other clade contained our *S. hominis* sequence together with its reference sequence. The variations were also observed in the multiple sequence alignment results.

Phylogenetic analysis is a modern way of testing hypotheses about a descent of species from a common ancestor (Persing et al., 2011). Sequences that have related genomes are easily influenced to detailed reconstruction of the genome evolution (Makarova & Koonin, 2007). Phylogenetic tree construction works on principles proposed by Charles Darwin that all living organisms descended from a common ancestor and that struggles for existence make offspring dissimilar from their parents. Based on this, organisms can be classified in taxonomic hierarchy (Persing et al., 2011).

Rooted phylogenetic trees often allow prediction of descendent. A total of 890bp were analysed in Figure 2, with each genus assigned. The taxonomic assignment of the sequences showed that the composition of human breast milk is dominated by cocci. Every genus was grouped with its members from the same root. However, *Staphylococcus* genus was split into two distant clades with about 80% bootstrap. The clade that consisted of *S. hominis* alone was bootstrapped 100% whereas the other comprising both *S. hominis* and *S. lugdunensi* had 64% bootstrap value. This implies that although both are of the same genus and/or species, they have differences in their molecular characteristics that lead to their possible evolutional variation. These differences were reported in their phenotypic characteristics as well (Abdalla et al., 2013). The clade containing the two *Staphylococci* species together with *Enterococci* and *Enterobacter* appeared to have evolved earlier than the other clade because it has a shorter length to their common ancestor.

Lack of deposition of sequences of bacteria isolated from human breast milk to GenBank or other online databases limited the phylogenetic comparison of our isolates to those found in

human breast milk from other countries and regions of the world. Based on the results obtained as discussed herein, *E. faecalis* and *S. hominis* are the most frequently isolated species from the human milk.



Phylogenetic tree showing the relative positions of identified isolates from human breast milk as inferred by the neighbour-joining method of partial 16SrDNA sequences. Bootstrap values for a total of 1000 replicates are shown at the nodes of the tree. References of the type strains used for comparison are given, as well as the accession numbers for all 16S rDNA sequences. Sequence divergence was at 0.2 scales.

Figure 2. Phylogenetic tree of 16S rDNA gene of bacteria isolated.

CONCLUSION

Three bacterial genera were successfully isolated and characterised from human breast milk using both phenotypic and molecular techniques. Based on the phenotypic characterisation, these isolates were found to metabolise a wide range of carbohydrates such as D-Maltose, Sucrose, D-Turanose, α-D-Glucose, D-Fructose, D-Mannose, D-Galactose, D-sorbitol and D-Mannitol. All the isolates were able to grow in acidic pH (5 and 6), 1% sodium lactate, 1%, 2% and 8% NaCl. The isolates' identities were further confirmed by molecular techniques, sequence and phylogenetic analyses. The knowledge of human milk commensal bacteria is limited especially in Malaysia. This study explored such commensal bacteria in expressed human milk of healthy lactating women. Isolation and identification of these bacteria from human milk served as the basis for further studies on benefit of these organisms towards human health such as in the area of biotherapeutics.

ACKNOWLEDGEMENTS

This study was funded by Fundamental Research Grants, Ministry of Higher Education, Malaysia, Vote number (5454244). We acknowledge the support of the Malaysian Society for Lactic Acid Bacteria during the sample collection exercise. We appreciate the effort and contributions of Faruku Bande (DVM) during sequence and phylogenetic analyses.

REFERENCES

Abdalla, N. M., Haimour, W. O., Osman, A. A., Sarhan, M. A., & Musaa, H. A. (2013). Antibiotics Sensitivity profile towards Staphylococcus hominis in Assir Region of Saudi Arabia. *Journal of Scientific Research*, *5*(1), 171-183.

Amusquivar, E., Ruperez, F. J., Barbas, C., & Herrera, E. (2000). Low arachidonic acid rather than alpha-tocopherol is responsible for the delayed postnatal development in offspring of rats fed fish oil instead of olive oil during pregnancy and lactation. *Journal of Nutrition*, *130*, 2855-2865.

Beasley, S. (2004). Isolation, identification and exploitation of lactic acid bacteria from human and animal microbiota. University of Helsinki, Finland.

Berlin, C. M., Denson Jr, H. M., Daniel, C. H., & Ward, R. M. (1984). Disposition of dietary caffeine in milk, saliva, and plasma of lactating women. *Pediatrics*, *73*(1), 59-63.

Bingen, E., Denamur, E., Lambert-Zechovsky, N., Aujard, Y., Brahimi, N., Geslin, P., & Elion, J. (1992). Analysis of DNA restriction fragment length polymorphism extends the evidence for breast milk transmission in Streptococcus agalaciae late-onset neonatal infection. *Journal of Infectious Diseases*, *165*, 569-573.

Buhnik-Rosenblau, K., Matsko-e, V., Danin-poleg, Y., Franz, C. M. A. P., Klein, G., & Kashi, Y. (2013). Biodiversity of Enterococcus faecalis based on genomic typing. *International Journal of Food Microbiology*, *165*, 27-34. doi:10.1016/j.ijfoodmicro.2013.04.009

Cabrera-Rubio, R., Collado, M. C., Laitinen, K., Salminen, S., Isolauri, E., & Mira, A. (2012). The human milk microbiome changes over lactation and is shaped by maternal weight and mode of delivery 1-4. *American Journal of Clinical Nutrition*, *96*, 544-551. doi:10.3945/ajcn.112.037382

Caroll, L., Osman, M., Davies, D. P., & McNeish, A. S. (1979). Bacteriological criteria for feeding raw breast-milk to babies on neonatal units. *Lancet*, *2*, 732-733.

Choi, S. S., Kim, Y., Han, K. S., You, S., Oh, S., & Kim, S. H. (2006). Effects of lactobacillus strains on cancer cell proliferation and oxidative stress in vitro. *Letters in Applied Microbiology.*, *42*, 452-458.

Deasyt, B. M., Real, M. C., Fitzgerald, G. F., Cogant, T. M., & Beresford, T. P. (2000). A rapid PCR based method to distinguish between Lactococcus and Enterococcus. *System and Applied Microbiology*, *23*, 510-522. doi:10.1016/S0723-2020(00)80025-9

Dubos, C., Vega, N., Carvallo, C., Navarrete, P., Cerda, C., Brunser, O., & Gotteland, M. (2011). Identification of Lactobacillus spp. in colostrum. *Archivos Latinoamericanos de Nutricion*, *61*(1), 66-68.

Dunne, C., O'Mahony, L., Murphy, L., Thornton, G., Morrissey, D., O'Halloran, S., … Collins, J. K. (2001). In vitro selection criteria for probiotic bacteria of human origin: Correlation with in vivo findings. *American Journal of Clinical Nutrition*, *73*, 386s-392s.

Eidelman, A. I., & Szilagyi, G. (1979). Patterns of bacterial colonization of human milk. *Obstetrics and Gynecology*, *53*, 550-552.

El-Mohandes, A. E., Keiser, J. F., Johnson, L. A., Refat, M., & Jackson, B. J. (1993). Aerobes isolated in fecal microflora of infants in the intensive care nursery: Relationship to human milk use and systemic sepsis. *American Journal of Infection Control*, *21*, 231-234.

El-Mohandes, A. E., Schatz, V., Keiser, J. F., & Jackson, B. J. (1993). Bacterial contaminants of collected and frozen human milk used in an intensive care nursery. *American Journal of Infection Control*, *21*, 226-230.

Emerenini, E. C., Afolabi, O. R., Okolie, P. I., & Akintokun, A. K. (2013). Isolation and molecular characterization of lactic acid bacteria isolated from fresh fruits and vegetables using nested PCR analysis. *British Microbiology Research Journal*, *3*(3), 368-377. Retrieved from www.sciencedomain.org

Felsenstein, J. (1985). Confidence limits on phylogenesis: An approach using the bootstrap. *Evolution*, *39*, 1596-1599.

Fernández, L., Langa, S., Martín, V., Maldonado, A., Jiménez, E., Martín, R., & Rodríguez, J. M. (2013). The human milk microbiota: Origin and potential roles in health and disease. *Pharmacological Research*, *69*(1), 1-10. doi:10.1016/j.phrs.2012.09.001

Fooks, L. J., Fuller, R., & Gibson, G. R. (1999). Prebiotics, probiotics and human gut microbiology. *International Dairy Journal*, *9*(1), 53-61. Retrieved from http://dx.doi.org/10.1016/S0958-6946(99)00044-8

Gregoret, V., Perezlindo, M. J., Vinderola, G., Reinheimer, J., & Binetti, A. (2013). A comprehensive approach to determine the probiotic potential of human-derived Lactobacillus for industrial use. *Food Microbiology*, *34*(1), 19-28. doi:10.1016/j.fm.2012.11.004

Hall, T. A. (1999). Bioedit: A user friendly biological sequence alignment editor and analysis program for windows 95/98/NT. *Nucleic Acid Symposium Series*, *41*, 95-99.

Heikkila, M. P., & Saris, P. E. J. (2003). Inhibition of Staphylococcus aureus by the commensal bacteria of human milk. *Journal of Applied Microbiology*, *95*(Huovinen 2001), 471-478. doi:10.1046/j.1365-2672.2003.02002.x

Holzapfel, W. H., Haberer, P., Snel, J., Schillinger, U., & Huis in't Veld, J. H. (1998). Overview of gut flora and probiotics. *International Journal of Food Microbiology*, *41*, 85-101.

Jackson, C. R., Fedorka-cray, P. J., John, B., & Barrett, J. B. (2004). Use of a genus- and species-specific multiplex PCR for identification of Enterococci. *Journal of Clinical Microbiology*, *42*(8), 3558-3565. doi:10.1128/JCM.42.8.3558

Kralj, S., van Geel-Schutten, G. H., Rahaoui, H., Leer, R. J., Faber, E. J., van der Maarel, M. J. E. C., & Dijkhuizen, L. (2002). Molecular characterization of a novel glucosyltransferase from Lactobacillus reuteri strain 121 synthesizing a unique, highly branched glucan with alpha-(1-->4) and alpha-(1-->6) glucosidic bonds. *Applied and Environmental Microbiology*, *68*(9), 4283-4291. doi:10.1128/AEM.68.9.4283-4291.2002

Law, B. J., Urias, B. A., Lertzman, J., Robson, D., & Romance, L. (1989). Is ingestion of milk-associated bacteria by premature infants fed raw human milk controlled by routine bacteriologic screening? *Journal of Clinical Microbiology*, *27*, 1560-1566.

Le Thomas, I., Mariani-Kurkdjian, P., Collignon, A., Gravet, A., Clermont, O., Brahimi, N., Gaudelus, J., … Bingen, E. (2001). Breast milk transmission of a panton-valentine leukocidin-producing Staphylococcus aureus strain causing infatile pneumonia. *Journal of Clinical Microbiology*, *39*, 728-729.

Lopez, I., Ruiz-larrea, F., Cocolin, L., Orr, E., Phister, T., Marshall, M., … Mills, D. A. (2003). Design and evaluation of PCR primers for analysis of bacterial populations in wine by denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology*, *69*(11), 6801-6807. doi:10.1128/AEM.69.11.6801

Makarova, K. S., & Koonin, E. V. (2007). Evolutionary genomics of lactic acid bacteria. *Journal of Bacteriology*, *189*(4), 1199-1208. doi:10.1128/JB.01351-06

Martín, R., Heilig, H. G. H. J., Zoetendal, E. G., Jiménez, E., Fernández, L., Smidt, H. and, & Rodríguez, J. M. (2007). Cultivation-independent assessment of the bacterial diversity of breast milk among healthy women. *Research in Microbiology*, *158*(1), 31-37.

Martín, R., Langa, S., Reviriego, C., Maræn, M. L., Fernández, L., Xaus, J., A., & Rodríguez, J. M. (2002). Transferencia de bacterias lácticas del intestino de la madre al intestino del lactante a través de la leche materna. In *Actas Del II Congreso Español de Lactancia Materna, Sevilla*.

Martin, R., Olivares, M., Marin, M. L., Fernandez, L., Xaus, J., & Rodriguez, J. M. (2005). Probiotic potential of 3 Lactobacilli strains isolated from breast milk. *Journal of Human Lactation*, *21*, 8-17.

Martineau, F., Picard, O. I. S. J., Ke, D., Paradis, S., Roy, P. H., Ouellette, M., & Bergeron, M. G. (2001). Development of a PCR assay for identification of staphylococci at genus and species levels. *Journal of Clinical Microbiology*, *39*(7), 2541-2547. doi:10.1128/JCM.39.7.2541

Morand, P. C., Billoet, A., Rottman, M., Eyrolle, L., Jeanne, L., Tazi, A., … Dumaine, V. (2009). Specific distribution within the Enterobacter cloacae complex of strains isolated from infected orthopedic implants. *Journal of Clinical Microbiology*, *47*(8), 2489. doi:10.1128/JCM.00290-09

Nasiraii, L. R., Tabatabaie, F., Alaeddini, B., Noorbakhsh, R., Heravi, R. A., & Afsharian, S. (2011). Investigation of lactobacilli from mother's breast milk. *African Journal of Microbiology Research*, *5*(13), 1581-1585. Retrieved from http://www.academicjournals.org/ajmr

Novak, F. R., da Silva, A. V., Hagler, A. N., & Figueiredo, A. M. S. (2000). Contamination of expressed human breast milk with an epidemic multiresistant Staphylococcus aureus clone. *Journal of Medical Microbiology*, *49*, 1109-1117.

Nueno-Palop, C., & Narbad, A. (2011). Probiotic assessment of Enterococcus faecalis CP58 isolated from human gut. *International Journal of Food Microbiology*, *145*, 390-394. doi:10.1016/j.ijfoodmicro.2010.12.029

Nuraida, L., Palupi, N. S., Bastomi, R. R., Priscilia, D., & Nurjanah, S. (2012). Evaluation of probiotics properties of lactic acid bacteria isolated from breast milk and their potency as starter culture. *International Journal of Food, Nutrition and Public Health*, *5*(1), 33-60. Retrieved from http://www.worldsustainable.org

Olivares, M., Diaz-Ropero, M. P., Martin, R., Rodriguez, J. M., & Xaus, J. (2006). Antimicrobial potential of four Lactobacillus strains isolated from breast milk. *Journal of Applied Microbiology*, *101*, 72-79.

Persing, D. H., Tenover, F. C., Yi-Wei, T., Nolte, F. S., Hayden, R. T., & Belkum, V. (2011). *Molecular Microbiology: Diagnostic principles and practice.* (2nd ed.). Washington, DC: ASM Press.

Prentice, A. (1996). Constituents of human milk. *Food and Nutrition Bulletin*, *17*(4). Retrieved from http://unu.edu/unupress/food/8F174e/8F174E04 htm

Pridmore, R. O., Berger, B., Desiere, F., Vilanova, D., Baretto, C., Pittet, A. C., … Schell, M. A. (2004). The genome sequence of probiotic intestinal bacterium Lactobacillus johnsonii NCC533. In *Proceedings of the National Academy of Sciences of the USA* (Vol. 101, pp. 2512-2517). doi:10.1073/pnas.0307327101

Reid, G. (2001). Probiotic agents to protect the urogenital tract against infection. *American Journal of Clinical Nutrition*, *73*, 437S-443S.

Saitou, N., & Nei, M. (1987). The neighbor-joining method : A new method for reconstructing phylogenetic trees. *Molecular Biology*, *4*(4), 406-425.

Scopesi, F., Ciangherotti, S., Lantieri, P. B., Risso, D., Bertini, I., Campone, F., … Serra, G. (2001). Maternal dietary PUFAs intake and human milk content relationships during the first month of lactation. *Clinical Nutrition*, *20*, 393-397.

Tamura, K., Dudley, J., Nei, M., & Kumar, S. (2007). MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*, *24*, 1596-1599.

Wagner, C. L. (2012). Human milk and lactation. *emedicine - medscape*. Retrieved from http://www.emedicine.medscape.com/article/1835675-overview#a0101

West, P. A., Hewitt, J. H., & Murphy, O. M. (1979). The influence of methods of collection and storage on the bacteriology of human milk. *Journal of Applied Bacteriology*, *46*, 269-277.

Wright, K. C., & Feeny, A. M. (1998). The bacteriological screening of donated human milk: Laboratory experience of British Paediatric Association's published guidelines. *Journal of Infection*, *36*, 23-27.