การตรวจหาแบคทีเรียที่มีประโยชน์ในน้ำนมมารดาและทดสอบ
ความสามารถในการยับยั้งแบคทีเรียก่อโรค

นางสาวยุพาวดี ชาวดง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

สาขาวิชาจุลชีววิทยาทางการแพทย์ (สหสาขาวิชา)

บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย

ปีการศึกษา 2554

DETECTION OF BENEFICIAL BACTERIA IN BREAST MILK AND ASSESSMENT OF THEIR ANTAGONISTIC ACTIVITY AGAINST BACTERIAL PATHOGENS

Miss Yupawadee Chaodong

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Medical Microbiology
(Interdisciplinary Program)
Graduate School
Chulalongkorn University
Academic Year 2011

Thesis Title	DETECTION OF BENEFICIAL BACTERIA IN BREAST MILK AND ASSESSMENT OF THEIR ANTAGONISTIC ACTIVITY AGAINST BACTERIAL PATHOGENS
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Field of Study	Medical Microbiology
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Accepted by the Graduate School, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

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ยุพาวดี ชาวดง : การตรวจหาแบคทีเรียที่มีประโยชน์ในน้ำนมมารดาและทดสอบความสามารถในการยับยั้งแบคทีเรียก่อโรค (DETECTION OF BENEFICIAL BACTERIA IN BREAST MILK AND ASSESSMENT OF THEIR ANTAGONISTIC ACTIVITY AGAINST BACTERIAL PATHOGENS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร. สมหญิง ธัมวาสร, 129 หน้า.

น้ำนมมารดาเป็นแหล่งอาหารที่สำคัญต่อทารกและมีบทบาทในการเริ่มต้นพัฒนาจุลินทรีย์ในลำไส้ของทารก ในการวิจัยครั้งนี้มีวัตถุประสงค์เพื่อตรวจแยกและพิสูจน์เอกลักษณ์ของเชื้อแบคทีเรียที่มีประโยชน์ในน้ำนมมารดา ได้แก่ lactobacilli, bifidobacteria และ streptococci และทดสอบคุณสมบัติของเชื้อเหล่านี้ในการยับยั้งเชื้อแบคทีเรียก่อโรค ได้แก่ enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), *Salmonella* Typhimurium, *Shigella flexneri*, *Vibrio cholerae*, *Helicobacter pylori* and methicillin-resistant *Staphylococcus aureus* (MRSA) ทำการเก็บตัวอย่างน้ำนมจากมารดาชาวไทยที่มีสุขภาพดี มีระยะการให้นมบุตรในช่วง 15-60 วัน (จำนวน 102 คน) พิสูจน์เอกลักษณ์ของ lactobacilli, bifidobacteria และ streptococci โดยการวิเคราะห์ลำดับนิวคลีโอไทด์บางส่วนของ 16S rRNA gene และทดสอบคุณสมบัติในการยับยั้งเชื้อแบคทีเรียก่อโรค โดยใช้ spot method ผลการตรวจแยกเชื้อพบ *Lactobacillus* ใน 37 ตัวอย่าง (36.27%) จำนวน 40 สายพันธุ์ ได้แก่ *L. gasseri*, *L. salivarius*, *L. fermentum*, *L. mucosae*, *L. rhamnosus, L. casei, L. plantarum* และ *L. oris* พบ *Bifidobacterium* ใน 31 ตัวอย่าง (30.39%) จำนวน 33 สายพันธุ์ ได้แก่ *B. longum*, *B. breve, B. psedocatenulatum*, *B. dentium* and *B. bifidum* และพบ *Streptococcus* ใน 17 ตัวอย่าง (16.67%) จำนวน 26สายพันธุ์ ได้แก่ *S. salivarius*, *S. lactarius*, *Streptococcus* sp., *Streptococcus mitis* และ *Streptococcus parasangius* ผลการตรวจหาดีเอ็นเอในตัวอย่างน้ำนม โดยวิธี พีซีอาร์ ตรวจพบดีเอ็นเอของ lactobacilli 94 ตัวอย่าง (92.16%) bifidobacteria 60 ตัวอย่าง (58.82%) และ streptococci 56 ตัวอย่าง (54.90%) ตามลำดับ การทดสอบการยับยั้งเชื้อETEC, EIEC, EPEC, EHEC and *S.* Typhimurium พบว่า *Lactobacillus* ทั้งหมดสามารถยับยั้งได้เล็กน้อย *Bifidobacterium* สามารถยับยั้งได้ไม่ชัดเจน และ *Streptococcus* ไม่มีความสามารถในการยับยั้ง ส่วนผลในการยับยั้งเชื้อ *V. cholerae* และ *S. flexneri* พบว่า *Lactobacillus* 13 สายพันธุ์ (Lac43, Lac44, Lac45, NL1, NL3, NL5, NL6, NL7, NL8, NL10, NL18, NL26 และ NL50) และ *Bifidobacterium* 11 สายพันธุ์ (Bif29, NB4, NB11, NB13, NB14, NB15, NB16, NB17, NB28, NB31 และ NB40) สามารถยับยั้งได้ชัดเจน นอกจากนี้ *Lactobacillus* 6 สายพันธุ์ (Lac 40, Lac41, NL26 NL50, NL52 และ NL53) และ *Streptococcus* 5 สายพันธุ์ (St10, St11, NL4, NL9 และ St27) สามารถยับยั้งเชื้อ MRSA ได้เล็กน้อย และ พบว่า *Bifidobacterium* 5 สายพันธุ์ (NB6, NB8, NB14, NB28 และNB31) สามารถยับยั้งเชื้อ *Helicobacter pylori* ได้อย่างชัดเจน *Lactobacillus, Bifidobacterium* และ *Streptococcus* มีความสามารถในการยับยั้งเชื้อแบคทีเรียก่อโรคเหล่านี้มีคุณสมบัติที่จะนำไปใช้เป็นโพรไบโอติกส์ในการยับยั้งเชื้อแบคทีเรียก่อโรค

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต ……………………………………………….
ปีการศึกษา 2554 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก…………………………...

5287245420 : MAJOR MEDICAL MICROBIOLOGY
KEYWORDS : BENEFICIAL BACTERIA/ BREAST MILK/ LACTOBACILLI/ BIFIDOBACTERIA/ STREPTOCOCCI

YUPAWADEE CHAODONG: DETECTION OF BENEFICIAL BACTERIA IN BREAST MILK AND ASSESSMENT OF THEIR ANTAGONISTIC ACTIVITY AGAINST BACTERIAL PATHOGENS. ADVISOR: ASSOC. PROF. SOMYING TUMWASORN, Ph.D., 129 pp.

Breast milk is an important nutrient for neonates and plays role in the initiation of the neonatal gut microbiota. This study aimed to isolate beneficial bacteria including lactobacilli, bifidobacteria and streptococci from breast milk and assess their antagonistic activity against bacterial pathogens including enterotoxigenic *E .coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), *Salmonella* Typhimurium, *Shigella flexneri*, *Vibrio cholerae*, *Helicobacter pylori* and methicillin-resistant *Staphylococcus aureus* (MRSA). Breast milk samples were collected from Thai healthy mothers (n=102) lactating at range 15-60 days. *Lactobacillus*, *Bifidobacterium* and *Streptococcus* species were identified by 16S rRNA gene sequencing and tested for their antagonistic activity against bacterial pathogens by agar spot method. Forty isolates of *Lactobacillus* which were *L. gasseri*, *L. salivarius*, *L. fermentum*, *L. mucosae*, *L. rhamnosus*, *L. casei*, *L. plantarum* and *L. oris* were recovered from 37 (36.27%) milk samples. Thirty-three isolates of *Bifidobacterium* including *B. longum*, *B. breve*, *B. psedocatenulatum*, *B. dentium* and *B. bifidum* were recovered from 31 (30.39%) milk samples. Twenty-six isolates of *Streptococcus* including *S. salivarius*, *S. lactarius*, *Streptococcus* sp., *Streptococcus mitis* and *Streptococcus parasangius* were presented from 17 (16.67%) milk samples. PCR assay demonstrated that DNAs of lactobacilli, bifidobacteria and streptococci were detected in 94 (92.16%), 60 (58.82%) and 56 (54.90%) of 102 breast milk samples, respectively. Antagonistic activity assay demonstrated that all *Lactobacillus*, *Bifidobacterium* and *Streptococcus* isolates had weak, partial as microcolony and no inhibition against ETEC, EIEC, EPEC, EHEC and *S.* Typhimurium, respectively. Thirteen *Lactobacillus* isolates (Lac43, Lac44, Lac45, NL1, NL3, NL5, NL6, NL7, NL8, NL10, NL18, NL26 and NL50) and 11 *Bifidobacterium* isolates (Bif29, NB4, NB11, NB13, NB14, NB15, NB16, NB17, NB28, NB31 and NB40) had strong inhibitory activities against *V. cholerae* and *S. flexneri*. Furthermore, six *Lactobacillus* isolates (Lac 40, Lac41, NL26 NL50, NL52 and NL53) and 5 *Streptococcus* isolates (St10, St11, NL4, NL9 and St27) weakly inhibited the growth of MRSA. In addition, 5 *Bifidobacterium* isolates (NB6, NB8, NB14, NB28 and NB31) had strong inhibitory activities against *H. pylori*. These *Lactobacillus*, *Bifidobacterium* and *Streptococcus* with antagonistic activity had potential for use as probiotics against bacterial pathogens.

Field of Study : Medical Microbiology Student's Signature ....

Academic Year : 2011 Advisor's Signature ....

ACKNOWLEDGEMENTS

I wish to express my deep gratitude to my thesis advisor, Associate Professor Somying Tumwasorn Ph.D., Department of Microbiology, Faculty of Medicine, Chulalongkorn University, my principal advisor for her valuable advice, encouraging guidance and indispensable help throughout the period of this study.

I am also grateful to my thesis committee, Associate Professor Ariya Chindamporn Ph.D., Department of Microbiology, Faculty of Medicine, Chulalongkorn University, the Chairperson and Associate Professor Sunee Korbsrisate, Ph.D., Department of Immunology, Faculty of Medicine, Mahidol University for their valuable comments and constructive criticisms.

Special thanks are given to Associate Professor Boosba Vivatvakin M.D., Faculty of Medicine, Chulalongkorn University and the staffs of well baby clinic, King Clulalongkorn Memorial Hospital for her kindness and help in specimen collection.

I would also like to thank all friends, and all staff members of Bacteriology Division, Department of Microbiology, Faculty of Medicine, Chulalongkorn University for their friendship, kindness and help in the laboratory during my study.

Finally, I would like to express my deepest gratitude to my parents for their love, encouragement, understanding and support during my study.

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LIST OF ABBREVIATIONS

ATCC	American type culture collection
bp	base pair
BHI	brain heart infusion
CFU	colony forming unit
CO_2	carbon dioxide
°C	degree celsius
DNA	deoxyribonucleic acid
DMST	Department of Medical Science, Thailand
DW	distilled water
EDTA	ethylenediamine tetraaceticacid
EHEC	enterohemorrhagic *Escherichia coli*
EIEC	enteroinvasive *Escherichia coli*
EPEC	enteropathogenic *Escherichia coli*
ETEC	enterotoxigenic *Escherichia coli*
et al.	et alii
g	gram
g/l	gram per liter
h	hour
HCl	hydrochloric acid
i.e.	id est
kb	kilobase
l	liter
M	molar
mg	milligram
mg/l	milligram per liter
min	minite(s)
ml	milliliter
mm	millimeter
MC	modified Columbia medium

MRS	deMan Rogosa Sharp
PCR	polymerase chain reaction
pmol	picomol
rpm	round per minute
16SrRNA	16S ribosomal ribonucleic acid
sec	second
TBE	tris-boric acid-EDTA
Tris	tris-(hydroxymethayl)-aminoethane
μg	microgram
μl	microliter
μM	micromolar
WHO	World Health Oganization

CHAPTER I

INTRODUCTION

Breast milk is an important source of nutritional requirements for the growing infant because of the composition of protective factors such as immunoglobulin A (IgA), immunocompetent cells, fatty acids, oligosaccharides, lysozyme and lactoferrin that improve and protect breast-fed infants against infectious diseases [1, 2]. In addition, breast milk is an important factor in the initiation and development of the infant gut microbiota because it is a source of microorganisms to the infant gut during breast-fed after birth. An infant fed breast milk about 800 Ml/d will ingest microorganisms about $1x10^5$-$1x10^7$ commensal bacteria [2] depending on hygiene and antibiotic use. In addition, it has been reported that the bacterial composition of the breast-fed infant flora reflected the bacterial composition of breast milk [3] Heikkila and Saris isolated commensal bacteria from breast milk, of which include four bacterial groups of staphylococci (64%), streptococci (30%), lactobacilli (10%), and enterococci (4%) [3]. Recently, it has been reported that bifidobacteria were also isolated from breast milk [4]. It was suggested that breast milk bacteria may be originated not only from external sources but also from the maternal gut microorganism. It was suspected that denditric cell that penetrated the gut epithelium take up bacteria directly from gut lumen. In addition, M cells on Peyer’s patch can phagocytose the gut microorganism. Once inside the cells, bacteria may be able to move from the intestinal mucosa to colonize distant mucosal surfaces, such as those of respiratory and genitourinary tracts, salivary and lachrymal glands and most significantly that of lactating mammary gland [5].

Specific strains of commensal bacteria in breast milk are beneficial bacteria, which include *Lactobacillus*, *Bifidobacterium*, and *Streptococcus*. *Lactobacillus* belongs to the lactic acid bacteria (LAB). They are Gram-positive rods or coccobacilli, catalase-negative, non-pathogenic and desirable members of the intestinal tract. They produce lactic acid as main end-product of the fermentation of carbohydrates [6]. *Lactobacillus* strains isolated from breast milk such as *L. gasseri*, *L. rhamnosus*, *L. plantarum* and *L. fermentum*, have beneficial effect of probiotic [7].

Bifidobacterium are Gram-positive polymorphic branched rods that occur singly, in chain or clumps. They are non-spore-forming, non motile, catalase-negative and produce acid but no gas from a variety of carbohydrates. They occur in animal and human habitats, in particular they have been isolated from feces, rumen of cattle, sewage, human vagina, dental caries and honey bee intestine [6]. Martin *et al*. recently reported the first isolation of bifidobacteria, i.e., *B. breve*, *B. adolescentis* and *B. bifidum* from breast milk [4]. Genus *Streptococcus* contains 60 species and its members are known for their pathogenicity, except *S. thermophilus*. It is Gram-positive, catalase-negative, non-motile, non-spore-forming, and used as starter for yoghurt production. The properties of this product attributed beneficial such as alleviation of symptoms of lactose intolerance and other gastrointestinal disorder [6]. Streptococci were the second most abundant bacteria in breast milk. Most common streptococci found in breast milk are *S. agalactiae, S. mitis*, *S. oralis, S. parasagis, S. peroris* and *S. salivarius*. It has been shown that *S. salivarius* could inhibit the growth of *Staphylococcus aureus* [3].

Commensal bacteria such as genera of *Lactobacillus*, *Bifidobacterium*, and *Streptococcus* are considered to be among the potential probiotic bacteria. Probiotics are “live microorganisms, which, when consumed in adequate amounts, confer a health benefit on the host” (FAO/WHO) [8]. The mechanisms of probiotics include remodeling of microbial communities, immunomodulation by up-regulation of anti-inflammatory factors, immunomodulation by suppression of pro-inflammatory factors, enhancement of immunity, effects on epithelial cell differentiation, proliferation, promotion of intestinal barrier function and suppression of pathogens [8]. Probiotic bacteria were able to suppress pathogens by producing acid, hydrogen peroxide or bacteriocin and small organic molecules [9]. It has been known for several decades that specific probiotic lactobacilli and bifidobacteria inhibit the growth of pathogen microorganisms such as *Escherichia coli*, *Salmonella enterica* , *Shigella sonnei*, *Helicobacter pylori* , *Staphylococcus aureus*, *Salmonella typhimurium*, *Yersinia enterocolitica* and *Clostridium perfringens* [7, 10]. *Lactobacillus* such as *L.reuteri* strains produces antimicrobial compound as reuterin that ability to inhibit the growth of enteric pathogens [11]. *Bifidobacterium* sp. produce bacteriocin that is able to inhibit the growth of food-borne pathogens such as

C. perfringens, E. coli, Salmonella and other human health-threatening pathogens such as the *H. pylori* [12]. *Streptococcus .thermophilus* can produce bacteriocin as thermophillins that inhibits *Clostridium tyrobutyricum* [13].

Identification of *Lactobacillus*, *Bifidobacterium* and *Streptococcus* employs conventional culture and molecular techniques. Culture technique is limited in the identification and quantification of these fastidious bacteria. Therefore culture-independent molecular method based on 16S rRNA genes, plays role in the identification of these bacteria. This study aims to isolate and identify beneficial bacteria such as *Lactobacillus, Bifidobacterium*, and *Streptococcus* in breast milk and investigate their antagonistic activity against bacterial pathogens of these bacteria.

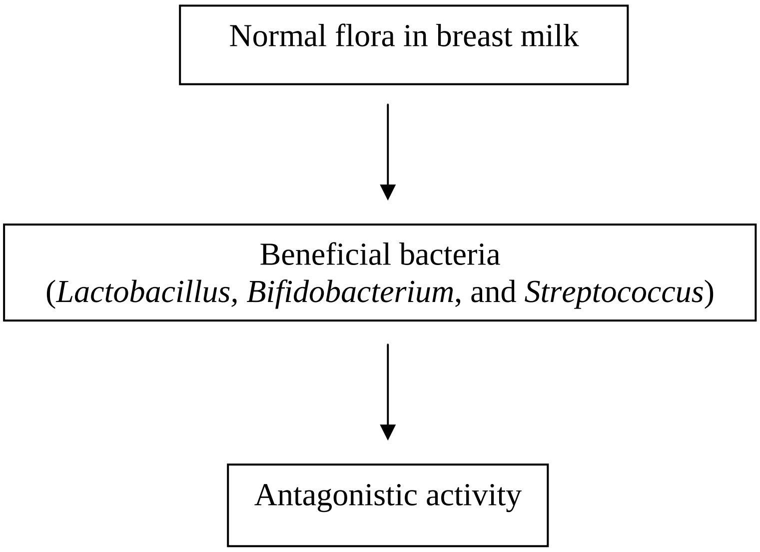
Hypothesis

Lactobacillus, Bifidobacterium, and *Streptococcus* isolated from breast milk confer antagonistic activity against bacterial pathogens.

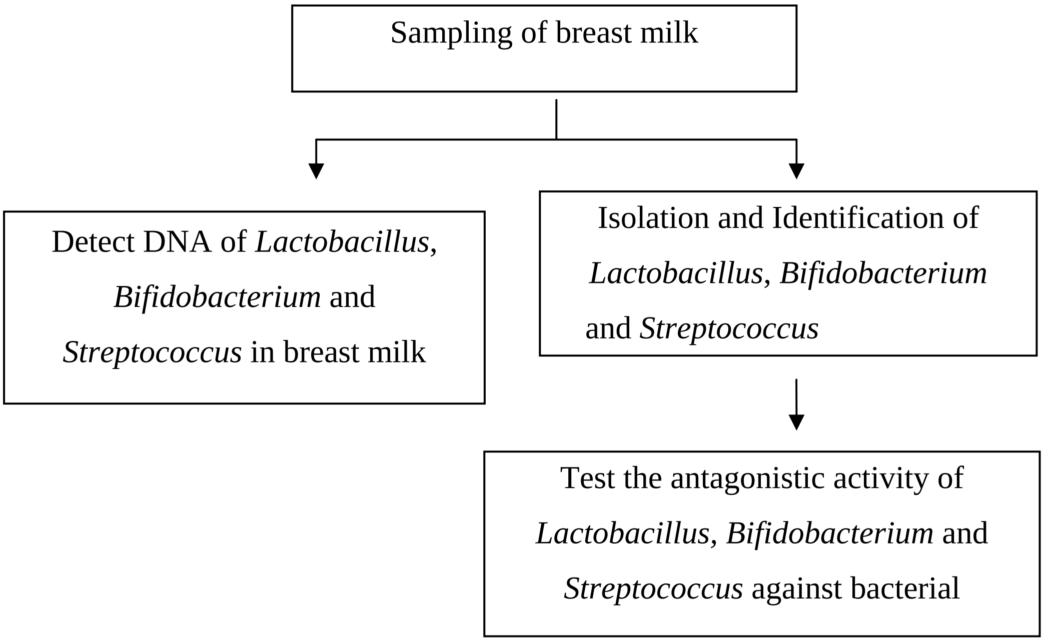
Objective

- Isolate and identify *Lactobacillus, Bifidobacterium*, and *Streptococcus* from breast milk.
- Detect DNA of *Lactobacillus*, *Bifidobacterium*, and *Streptococcus* in breast milk using PCR method.
- Test the antagonistic activity of *Lactobacillus*, *Bifidobacterium*, and *Streptococcus* isolates from breast milk to bacterial pathogens.

Conceptual framework



Workflow



CHAPTER II

LITERATURE REVIEWS

1. Breast milk

Breast milk is a unique, species-specific, complex nutritive fluid with immunologic and growth-promoting properties as follows. Immunoglobulin A (IgA) is an important protective factor against infection especially when the infant has limited defense against ingested pathogens. Lactoferrin in breast milk is an iron-binding protein which binds to iron, thus making it unavailable to pathogenic bacteria. Lysozyme which enhance sIgA bactericidal activity against gram-negative organisms. Oligosaccharides which intercept bacteria and form harmless compounds that the baby excretes. Lipids are known to be potent antimicrobial/microbicidal agents *in vitro* and to kill enveloped viruses, Gram-positive and Gram-negative bacteria and fungi on contact. Mucins which are present on milk-fat globule membrane. Mucins adhere to bacteria and viruses and help eliminate them from the body. In addition, breast milk also contains growth modulators such as epidermal growth factor (EGF), nerve growth factor (NGF), insulinlike growth factors (IGFs), interleukins, Transforming growth factor (TGF)-alpha and TGF-beta [14, 15]. The composition of breast milk that improve and protect breast-fed infants against infectious diseases [3, 5]. Breast milk consists of commensal bacteria such as genus *Staphylococcus* sp., i.e. *S. epidermidis, S. hominis, S. capitis* and *S. areus* that bacterial flora of the maternal skin. *Streptococcus* sp., i.e. *S. salivarius, S. mitis, S. parasanguis* and *S. peroris,* genus *Lactobacillus* sp., i.e. *L. gasseri, L. rhamnosus, L. acidophilus, L. plantarum* and *L. fermentum*, genus *Enterococcus* sp., i.e. *E. faecium* and *E. faecalis* [5] and genus *Bifidobacterium* sp., i.e. *B. adolescentis*. *B. longum, B. breve* and *B. bifidum* [4].

It was suggested that breast milk bacteria may be originated not only from external sources but also from the maternal gut microorganism. It was suspected that denditric cell that penetrated the gut epithelium take up bacteria directly from gut lumen [16]. In addition, M cells on Peyer's patch can phagocytose the gut microorganism. Once inside the cells, bacteria may be able to move from the intestinal mucosa to colonize distant mucosal surfaces, such as those of respiratory and genitourinary tracts, salivary and lachrymal glands and most significantly that of lactating mammary gland shown in Figure 1 [5, 17].

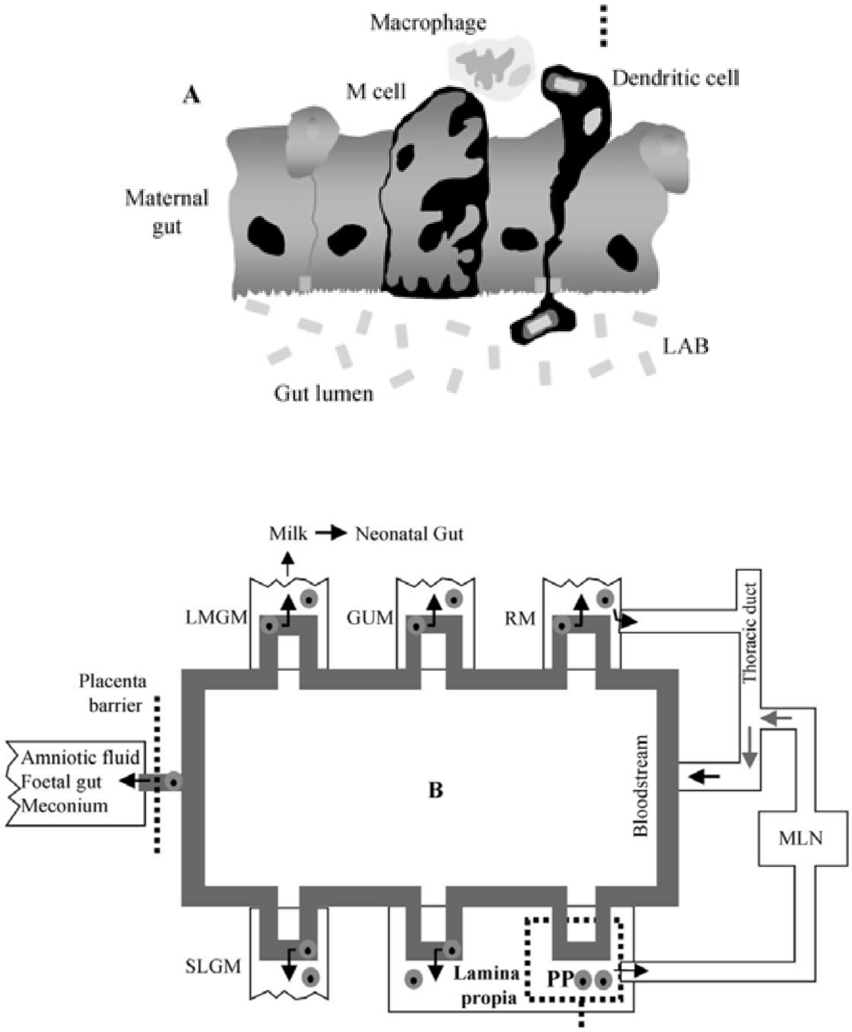


Figure 1. The hypothetical model to explain how some bacterial strains could be presented in breast milk. (A) dendritic cell that penetrated the gut epithelium take up bacteria directly from gut lumen. (B) M cells on Peyer's patch can phagocytose the gut microorganism. Abbreviations: GUM, genitourinary tract mucosa; LMGM, mucosa of the lactating mammary gland; MLN, mesentric lymph node; PP, Peyer patches and associated lymphoid tissue; RM, respiratory tract mucosa; SLGM, mucosa of the salivary and lacrimal glands.

The commensal bacteria exhibited in breast milk was suggested to be a major factor in the initiation and development of the infant gut microbiota [5]. In addition, it has been reported that the bacterial composition of the breast-fed infant flora reflected the bacterial composition of breast milk [3]. Beneficial bacteria were presented in breast milk such as *Lactobacillus, Bifidobacterium* and *Streptococcus*.

1. The genus *Lactobacillus*

1.1 Background of lactobacilli

The genus *Lactobacillus* belongs to the phylum *Firmicutes,* class *Bacilli*, order, *Lactobacillales,* family *Lactobacillaceae.* The first species of genus *Lactobacillus* was isolated from milk as *Lactobacillus delbruceckii* by Leichman (1896). After the few years, Moro (1900) recovered was *Lactobacillus acidophilus* by culture from the breast-fed infant feces. *Lactobacillus casei* is the name of lactobacilli isolated from cheese, milk and dairy product by Orla-jensen (1904). Lauer and Kandler (1980) isolated *Lactobacillus gasseri* from human mouth, vagina and intestinal tract of man. In 1953 Rogosa *et al*. isolated *Lactobacillus salivarius* from mouth and intestinal tracts which similar *Lactobacillus murinus* isolated by Heijenoort *et al* [18]. Currently, genus *Lactobacillus* consists of more than 175 species of which 19 species are of research interest as probiotics as shown in Table 1.

Table 1. *Lactobacillus* species of research interest as probiotics [19].

Species	**Original isolated**	**Mol% G+C**
L. acidophilus	Breast-fed infant feces, intestinal tract of human and animal	32-37
L. agilis	Sewage , human intestinal tract	34-36
L. aviarius	Human and chicken intestinal tract	34-36
L. amylovorus	Cattle waste-corn fermentation	40-41

Table 1. *Lactobacillus* species of research interest as probiotics [19] (Continued)

Species	**Original isolated**	**Mol% G+C**
L. brevis	Milk, cheese, feces, mouth, gastrointestinal tract of human	44-47
L. casei	Milk, cheese, dairy product, human intestinal tract, mouth, vagina	45-47
L. crispatus	Human feces, vagina	35-38
L. delbrueckii subsp. *bulgaricus*	Yoghurt and cheese	49-51
L. galinarum	Human intestinal tract, vagina	36-37
L. gasseri	Human mouth and vagina	33-35
L. johnsonii	Human vaginal discharge and blood clot	35-37
L. murinus	Intestinal tract of mice and rat	43-44
L. hamsteri	Feces of hamster	33-35
L. intestinalis	Intestine of human and animal	33-35
L. plantarum	Dairy products and environment	44-46
L. reuteri	Feces of human and animal, meat products	40-42
L. ruminis	Rumen of cow and sewage, human feces	44-47
L. salivarius	Mouth and intestinal tract of human and hamster	34-36
L. rhamnosus	Intestine, mouth and vagina of human, dairy environment	45-47

1.2 Biology of lactobacilli

Lactobacilli are Gram-positive rod or coccobacilli shape, non-spore-forming, non-motile microorganisms. They are fermentative, microaerophylic and chemo-organotrophic, requiring rich media to grow. They are catalase negative, even if psedocatalase activity can sometime be present in some strains. Members of the genus *Lactobacillus* can be selected on solid culture media that have an acidic pH (e.g. Rososa SL agar). While many *Lactobacillus* strains used in the diary industry can be culture under microanerophilic, or aerobic conditions, intestinal isolates proliferate best under anaerobic conditions [18].

The genus *Lactobacillus* includes more than 175 validly described species. They are found in environments where carbohydrates are available such as food (dairy products, fermented meat, sour doughs, vegetables, fruits, beverages), respiratory, GI and genital tracts from human and animals and in sewage and plant material [20]. They are growth at temperature rage 2-53 °C and optimum generally at 30-40°C. Optimum pH for growth at 5.5-6.2 and growth generally occur pH 5.0 or less. The G+C content of DNA 32-53 mol%. They are complex nutritional requirement for growth found as amino acid, vitamin, peptide, salt, nucleic acid, fatty acid or fatty acid esters and fermentable carbohydrates [18]. The main of fermentation pathways are obligately homofermentative; lactobacilli are able degrade hexoses to lactic acid by the Embden-Meyerhof pathway (EMP), facultatively heterofermentative; lactobacilli degrade hexoses to lactic acid by the EMP and are also able degrade pentose or glucose to aldolase and phosphoketolase, finally, obligately heterofermentative; lactobacilli are degrade hexoses to lactate, ethanol or acetic and CO_2 by the phosphogluconate pathway [21]. Genus *Lactobacillus* are identify base on metabolic characteristics, phylogenetic grouping, genome GC. In addition, the fastest way to identify lactobacilli used molecular method such as comparison of 16S rDNA gene sequences, the method shows that the V1, V2 and V3 regions contain the species-specific information, 16S, 23S and 5S rRNA genes are arranged within an operon on the bacterial chromosome [22, 23].

1.3 Beneficial effect of lactobacilli

Lactobacilli are lactic acid bacteria (LAB) and are normally consumed in the form of yoghurt, fermented milk or fermented food. They are colonized in the human large intestine and suggested a beneficial role for the host. In addition, some strains of *Lactobacillus* are plays role of probiotics. These effects are likely to involve both microbe-microbe and microbe-host interaction such as anti-microbial effects, immunomodulatory properties and gastrointestinal benefits. The beneficial effect of lactobacilli summarized shown in Table 2.

Table 2. The summary of beneficial effect of lactobacilli

Beneficial effect	Study summary	Reference
Prevention of gastrointestinal disease	Lactobacillus GG compared with a placebo product has been shown to significantly reduce the risk of in particular rotavirus gastroenteritis (2.2% compared with 17%, $P = 0.02$) in hospitalized children	[24]
	Lactobacillus GG and *L. acidophilus* were evaluated and compared with placebo for the prevention of side-effects in the treatment of *H. pylori*	[25]
	L. rhamnosus 19070-2 and *L. reuteri* DSM 12246, ameliorated acute diarrhea in hospitalized children and reduced the period of rotavirus excretion.	[26]
	L. paracasei could act as a potential barrier to prevent *S. aureus*- associated injury	[27]

Table 2. The summary of beneficial effect of lactobacilli (Continued)

Beneficial effect	**Study summary**	**Reference**
Cancer prevention	Specific strain *L. casei* showing significantly (*P*= 0.03) postponed tumor recurrence in 48 patients after removal of one or more bladder tumors.	[28]
Cholesterol reduction	The tablets contains *L. bulgaricuus* ATCC 33409 and *L. acidophilus* ATCC 4962 showing serum cholesterol reduced from 5.7 to 5.3 mmol/L after 7 wk (*P*< 0.05) in the 23 pilots.	[29]
	Specific strain of *L. acidophilus* contains with buffalo fermented milk showing serum cholesterol reduced to 12-20% after 1 month	[30]
Anti- microbial effects	*L. salivarius* UCC118 produce bacteriocin for against *Listeria monocytogenes* the invasive food-borne pathogen	[31]
	L.gasseri CECT5714 isolated from breast milk produce anti-microbial compound to inhibit *E.coli*, *Salmonella* spp. and *Listeria monocytogenes*	[32]

2. The genus *Bifidobacterium*

2.1 Background of bifidobacteria

The genus *Bifidobacteriun* belongs to the phylum *Actinobacteria,* class *Actinobacteria*, subclass *Actinobacteridae*, order *Bifidobacteriales,* family *Bifidobacteriaceae*. Bifidobacteria were first isolated from the breast-fed infant feces in 1899, by Henri Tissier, and were designated *Bacillus bididus* [33]. Even though Orla-Jensen proposed the genus *Bifidobacterium* in 1924[34], bifidobacteria were classified into other taxonomic group, such as *Bcillus bifidus* (1900), *Bacteroides bifidus* (1923 to 1934, in the 1st to 4th editions of Bergey's Manual of Systematic Bacteriology Bergey's Manual) and *Lactobacillus bifidus* (1939 to 1957, in the 5th to 7th editions of Bergey's Manual), for several decades. In 1973, Poupard *et al.*[35], and subsequently the 8th edition of Bergey's Manual [36], reclassified them as a separate taxon and designated the genus *Bifidobacterium*. Currently, genus *Bifidobacterium* contains 31 species that have been isolates from intestine of humans, animal and insects, and also from human dental caries and raw milk shown in Table 3**.**

Table 3. Currently defined species of the genus *Bifidobacterium* [37]

Species	Subspecies	Original isolated	%G+C^a	Reference
B. adolescentis		Intestine of adult	59.6 _ 0.8	[38]
B. angulatum		Human feces	59.0 _ 0.1	[39]
B. animalis	*B. animalis* subsp. *Animalis*	Animal feces	60.1 _ 0.3	[40]
	B. animalis subsp. *lactis*	Yogurt	61.9	[41]
B. asteroides		Intestine of honeybee	59.0	[42]
B. bifidum		Infant feces	62.3	[43]
B. bombi		Intestine of bumblebee	47.2	[44]
B. boum		Rumen of cattle	60 _ 0.2	[45]
B. breve		Intestine of infant	58.8 _ 0.4	[46]

a; Mean _ SD. , ND, not determine

Table 3. Currently defined species of the genus *Bifidobacterium* (Continued)[37]

Species	**Subspecies**	**Original isolated**	**%G+C^{a}**	**Reference**
B. catenulatum		Intestine of adult	54.0 _ 0.2	[39]
B. choerinum		Porcine feces	66.3 _ 0.2	[45]
B. coryneforme		Intestine of honeybee	ND	[47]
B. crudilactis		Raw milk	56.4	[48]
B. cuniculi		Feces of rabbit	64.1 _ 0.4	[45]
B. dentium		Human dental caries	61.2 _ 0.4	[39]
B. gallicum		Human feces	ND	[49]
B. gallinarum		Chicken cecum	65.7 _ 1.5	[50]
B. indicum		Intestine of honeybee	60.0	[42]
B. longum	*B. longum* subsp. *Infantis*	Intestine of infant	60.5 _ 0.3	[46]
	B. longum subsp. *Longum*	Intestine of adult	60.8 _ 0.8	[46]
	B. longum subsp. *Suis*	Porcine feces	62.0	[51]
B. magnum		Rabbit feces	60.0 _ 0.6	[52]
B. merycicum		Bovine rumen	ND	[53]
B. minimum		Sewage	61.5	[47]
B. pseudocatenulatum		Infant feces	57.5 _ 0.3	[45]
B. pseudolongum	*B. pseudolongum* subsp. *Globosum*	Bovine rumen	63.8 _ 0.4	[47]
	B. pseudolongum subsp. *Pseudolongum*	Porcine feces	59.5 _ 0.4	[54]

a; Mean _ SD. , ND, not determine

Table 3. Currently defined species of the genus *Bifidobacterium* (Continued)[37]

Species	**Subspecies**	**Original isolated**	**%G+C^a**	**Reference**
B. pyschraerophilum		Porcine feces	59.2	[55]
B. pullorum		Chicken feces	67.5 _ 0.4	[56]
B. ruminantium		Bovine rumen	ND	[53]
B. saeculare		Rabbit feces	ND	[57]
B. scardovii		Human blood	ND	[58]
B. subtile		Sewage	61.5	[59]
B. thermophilum		Porcine feces	60.0	[40]
B. thermacidophilum	*B. thermacidophilum* subsp. *porcinum* *B. thermacidophilum* subsp. *Thermacidophilum*	Sewage	ND	[60]

a; Mean _ SD. , ND, not determine

2.2 Biology of bifidobacteria

Bifidobacteria are anaerobic bacteria. They are Gram-positive, polymorphic branched rods that occur singly, in chains or clumps. *N*-acetlylamino-sugar, Ca^{2+} ions, or amino acid (alanine, aspartic acid, glutamic acid, and serine) has revealed of bifidobacteria morphology, that the absence or low concentrations of in growth media exclusively induce the bifid shape of bifidobacteria [61]. They are non-motile, non-spore-forming and non-filamentous. Bifidobacteri produce acid but not gas from a variety of carbohydrates. They are catalase negative, with some exception, *Bifidobacterium incidum* and *Bifidobacterium asteroids* when grown in presence of air [6]. Optimum growth temperature 37-41 °C, the strains isolated from human intestine growth at 37-38 °C and isolated from animal intestine growth at 41 °C. The high growth temperature 46 °C and low growth temperature 25-28 °C. Optimum pH for growth 6.5-7.5 and could not growth at lower than pH 4.5-5.0 or higher than pH 8.0-8.5 [62]. Bifidobacteria has with a high G+C content 55 to 57 mol % [6, 63, 64]. They present in many habitats such as feces of human and animal, rumen of cattle, sewage, human vagina, dental caries and honey bee intestine [6, 37]. Recently, has isolated bifidobacteria from breast milk [4]. Bifidobacteria degrade hexose through fructose-6-phosphate pathway by using fructose-6-phosphate phosphoketolase, adolase and glucose-6-phosphate dehydrogenase enzyme and the end product is lactic acid and acetic acid [61]. The taxonomic character for identify on genus level was considered with the enzyme [65]. Detection and identification of bifidobacteria were approached for three principal such as culturing method using a selective media for selection and identification, culture absence molecular methods for detection and molecular method for identification and differentiation. Selective media developed for bifidobacteria contain with antibiotic, it was inhibitory to some bifidobacteria [66, 67]. Beerens (1990) improved *Bifidobacterium*-selective medium, columbia medium by addition of 5 g/l glucose, 5 g/l cysteine hydrocroride, 5 g/l agar, 5 ml propionic acid and adjusted to pH 5.0 was both elective and selective for all species of *Bifidobacterium* [68]. Molecular method has highly enhanced approaches for detection, differentiation and identification of bifidobacteria. The molecular tools for use and development of these bifidobacteria such as AP-PCR (arbitrarily primed PCR) use of a single indiscriminate primer to gain of banding patterns for strain-specific; the subject can be to reproducibility problems [69], ARDRA (amplified rRNA gene restriction analysis) i.e., RFLP analysis of the *ldh* gene [70] or RFLP analysis of the 16S rRNA gene [71], PFGE (pulsed-field gel electrophoresis) use of a band profile analysis of complete genome by use of scarce-cutting enzymes [72] and 16S rRNA gene sequence analysis [73, 74].

2.3 Beneficial effect of bifidobacteria

Bifidobacteria are bacteria in the human large intestine and suggested a helpful for the host. There shown significantly event higher in the un-weaned infant gut more than in adults, they may a more important role in gut microbiota development than in other gut function. The properties of bifidobacteria in the large intestine of human including interactions with other gut microbes, production of vitamins including group of B vitamins, modulation of convinced bacterial groups that may be baneful to the host, production antimicrobial compounds found as organic acid [62] , iron-scavenging compounds [75] and bacteriocin [76, 77]. The function of bifidobacteria in the intestine may be in protection against some immune-based disorders, as previous studies have shown them to stimulate a host innate immune response [78, 79]. Numerous studies have suggested that the human health benefits is associated possession of bifidobacteria in the human large intestine such as prevention of diarrhea, establishment of a healthy microflora in premature infants, colon regularity, lactose intolerance, cholesterol reduction and immunostimulatory effects. These potential health benefits were summaries in Table 4.

Table 4. Summary of potential health benefits of bifidobacteria

Health benefit	**Study summary**	**Reference**
Prevention of diarrhea	*B. bifidum* and *B. longum* subsp. *infantis* showing a protective effect against rotaviral diarrhea, a statistically significant (*P* _ 0.001)	[80]
	B. breve showing a protective effect against rotaviral diarrhea but non-statistically significant	[81]
	B. bifidum showing a protective effect reduced shedding of rotavirus (*P* _ 0.01)	[82]
	B. animalis subsp. *lactis* showing protective effect against all forms of diarrhea and a higher titer of antirotaviral antibodies in the feces, statistically significant (*P* _ 0.01)	[83]

Table 4. Summary of potential health benefits of bifidobacteria (Continued)

Health benefit	Study summary	Reference
Prevention of diarrhea	*B. bifidum* and *Streptococcus thermophilus* showing protective effect reduced shedding of rotavirus , statistically significant (*P* _0.035)	[84]
	B. animalis subsp. *lactis* showing protective effect but a non-statistically significant	[85]
	B. breve and *S. thermophilus* showing a reduced severity of diarrhea episodes over a 5-month period (*P* _ 0.01)	[86]
	B. animalis and 4 species of *Lactobacillus* showing reduced the incidence of necrotizing enterocolitis (*P* _ 0.05)	[87]
Establishment of a healthy microflora in premature infants	*B. breve* showing resulted in establishment of a bifidobacterial flora in the majority of infants during the first week of life, whereas it took the control group several weeks, with only 3 of 9 infants showing bifidobacteria by week 7	[88]
	B. breve showing reduced fecal butyric acid levels, but only in the subgroup of infants that weighed 2,500 g (*P* _ 0.05)	[89]

Table 4. Summary of potential health benefits of bifidobacteria (Continued)

Health benefit	Study summary	Reference
Colon regularity	*B. animalis* subsp. *lactis* and yogurt cultures showing some reduce in colonic transit times (*P* _ 0.05)	[90]
	B. animalis subsp. *lactis* and yogurt cultures showing no statistically significant reduce in colonic transit times	[91]
	Supplementation with *B. animalis* subsp. *lactis* showing some reduce in colonic transit times (*P* _ 0.05)	[92]
	B. animalis subsp. *lactis* showing a reduce in colonic transit after 2 weeks (*P* _0.001)	[93]
Lactose intolerance	*B. longum* showing some reduce in breath hydrogen (*P* _ 0.05)	[94]
	B. animalis subsp. *lactis* and yogurt cultures showing some reduce in symptom scores (*P* _ 0.05)	[95]
Cholesterol reduction	*B. animalis* subsp. *lactis* and *L. acidophilus* showing some reduce in serum cholesterol levels (*P* _ 0.05)	[96]

Table 4. Summary of potential health benefits of bifidobacteria (Continued)

Health benefit	Study summary	Reference
Cholesterol reduction	Yoghurt containing with *L. acidophilus* 145, *B. longum* 913 and 1% oligofructose (synbiotic). showing no reduction in total cholesterol but an increase in high-density lipoprotein (HDL) levels (*P* _ 0.001)	[97]
	B. longum and *L. acidophilus* showing did not affect cholesterol levels	[98]
Immunostimulatory effects	*B. bifidum* showing reduce in CD4_ T cells in the spleen and colon (*P* _ 0.05)	[99]
	B. longum subsp. *infantis* showing some reduce in the proinflammatory cytokines IFN-, TNF-, and IL-12	[100]
	B. longum showing some increase in mucosal IgA (*P* _ 0.05)	[101]
	B. animalis subsp. *lactis* and *Lactobacillus paracasei* showing no statistically significant changes in cytokine levels	[102]
	B. animalis subsp. *lactis* showing some increase in the anti-inflammatory cytokine IFN- and in phagocytic activity (*P* _ 0.05)	[78]

Table 4. Summary of potential health benefits of bifidobacteria (Continued)

Health benefit	Study summary	Reference
Immunostimulatory effects	*B. longum*, inulin, and fructooligosaccharides showing some decrease in expression of genes encoding human proinflammatory cytokines (*P* _ 0.05)	[103]
	B. longum subsp. *infantis* showing reductions in symptom scores and in the ratio of IL-10 to IL-12 (anti-inflammatory to proinflammatory cytokines), normalized to that of healthy individuals	[104]
Cancer prevention	Heat-killed *B. infantis* showing some reduce in the incidence of tumors (*P* _ 0.01) in mice	[105]
	B. longum showing some decrease in carcinogenesis reduced aberrant crypt foci (*P* _ 0.05) and a significant decrease following co-supplementation with *B. longum* and inulin (*P* _ 0.001) in mice	[106]
	B. animalis subsp. *lactis* showing a significant reduce in carcinogen-induced colonic neoplasms (*P* _ 0.001) in mice	[107]
	B. animalis subsp. *lactis*, *L. rhamnosus*, and inulin showing some improve in epithelial barrier function and cell toxicity only in polypectomized patients (*P* _ 0.05) in cancer or polypectomized patients	[108]

3. The genus *Streptococcus*

3.1 Background of streptococci

The genus *Streptococcus* belongs to the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales* family *Streptococcaceae*. This species originally isolated from suppurative lesions in human. Since 1874, Billroth he observe to chain-forming cocci in wounds and applied the term “streptococcus” as organisms to designate their morphological arrangement. A few years later, Rosenbach (1884) first used the word Streptococcus in the generic sense and describe the species *Streptococcus pyogenes* which is now the type species of the genus. The species group recognized to genus *Streptococcus* in currently such as pyogenic, mitis, salivarius, anginosus, mutans and bovis (Table 5). Genus *Streptococcus* including about 60 species and a number of them is known for their pathogenicity. The beneficial streptococci found as salivarius group. *Streptococcus thermophilus* species is contain in the group of lactic acid bacteria (LAB). It is one the microorganisms using in dairy product and most commercially important of all LAB. It is association with *L. delbrueckii* subsp. *Bulgaricus*. Since 1984 Farrow and Collins reclassify *S. thermophilus* as *S. salivarius* subsp. *thermophilus* but the definition of its status of separate species have definitively been established by Schleifer *et al*. (1991) with the name of *Streptococus thermophilus*

Table 5. The species groups in genus *Streptococcus* [109, 110]

Group	**Species**
Pyogenic	*S. pyogenes. S. agalactiae, S. canis, S.dysgalactiae, S. equi, S. parauberis, S. iniae, S. parauberis, S.porcinus, S. uberis*
Mitis	*S. gordonii, S. mitis, S. oralis, S. parasanguis, S.pneumoniae, S. sanguis*
Salivarius	*S. salivarius, S. thermophilus, S. vestibularis*
Anginosus	*S. anginosus, S.constellatus, S. intermedius*
Mutans	*S. mutans, S. cricetus, S. downei, S. macacae, S. rattus, S. sobrinus*
Bovis	*S. vobis, S. alactolyticus, S. equinus*

3.2 Biology of streptococci

Streptococci are Gram-positive cocci, which may be spherical or ovoid in shape and are usually arranged in chain or pairs. They are non motile and do not formendospores. These streptococci are growth in facultative anaerobe, but some strains require CO_2. They are catalase-negative and homofermentative. The growth temperature at 10-45 °C and low of G+C DNA content 35-43 mol%. The streptococci are found an the mucous membranes of the mouth, upper respiratory tract, alimentary tract and human and animal skin [111]. In addition, lactic acid streptococci were recovered in fermented milk such as yoghurt and cheese [112, 113]. *Streptococcus thermophilus* detected by specific amplified of *lacZ* gene, rapid and reliable PCR-based technique [114].

3.3 Beneficial effect of streptococci

Genus *Streptococcus* is considered to be lactic acid bacteria (LAB). *Streptococus thermophilus* is an important LAB used for the food industry such as used for the manufacture of dairy product, used for as starter culture combination with *Lactobacillus delbrueckii* subsp. *bulgaricus* for production of yoghurts [113] and usage in cheese production i.e., Swiss cheese, Brick cheese, Parmesan, Provolone, Mozzarella and Asiago [112]. *Streptococcus thermophilus* is ability to survive in gastrointestinal tract and moderately adhere to intestinal epithelial cells [115]. The beneficial effect of *Streptococcus thermophilus* has been shown as positive effects on diarrheas in young children, enterocolitis in premature neonates and inflammatory gut disease [116]. Furthermore, it has shown produce antioxidants [117], stimulate the gut immune system [116], alleviate the risk of certain cancer and improve lactose digestion in lactose intolerant individuals [118]. In addition, it has shown inhibits *Clostridium tyrobutyricum* by production of bacteriocin [13].

4. Antimicrobial compound of *Lactobacillus, Bifidobacterium* and *Streptococcus*

Antimicrobial compound is produced by lactic acid bacteria (LAB) such as *Lactobacillus, Bifidobacterium* and *Streptococcus*. The antimicrobial compound is classified as low-molecular-mass (LMM) compounds such as organic acid, hydrogen peroxide (H_2O_2), carbon dioxide (CO_2), diacetyl (2,3-butanedione) and high-molecular-mass (HMM) compounds like bacteriocins [119].

4.1 Organic acid

The organic acid is product by LAB fermentation and the character of organic acid associate with accumulation of organic acids and the accompanying reduction in pH. The type of organic acid found as lactic acid, acetic acid and propionic acid. Lactic acid is the main metabolite of LAB fermentation and the boundary of the dissociation depends on pH. It is toxicity to many bacteria, fungi and yeasts. At pH 5.0 lactic acid was inhibit to spore-forming of bacteria but was no effective against yeasts and moulds [120]. Acetic acid and propinonic are more effective of antimicrobial than lactic acid because their have higher pKa values (lactic acid 3.08, acetic acid 4.75, and propionic acid 4.87), and their have higher percent of undissociated acids than lactic acid at a given pH [121]. Acetic acid was more inhibition growth of *Listeria monocytogenes* [122] and *Bacillus cereus* [123] more than acetic acid.

4.2 Hydrogen peroxide

The antimicrobial effect of H_2O_2 may result from the oxidation of sulfhydryl groups causing denaturing of a number of enzymes, and from the peroxidation of membrane lipids thus the increased membrane permeability. It has been reported that H_2O_2 produce by *Lactobacillus* and *Lactococcus* strains could be inhibit *Staphylococcus aureus, Pseudomonas* sp.[124].

4.3 Carbon dioxide

Carbon dioxide CO_2 may properties in creating an anaerobic environment which inhibits enzymatic decarboxylations and the accumulation of CO_2 in the membrane lipid bilayer may cause a dysfunction in permeability. CO_2 can inhibit the growth of many food spoilage microorganisms, especially Gram-negative psychrotrophic bacteria [125].

4.4 Diacetyl (2,3-butanedione)

Diacetyl is produced by strains within all genera of LAB by citrate fermentation. It inhibits the growth of Gram-negative bacteria more than Gram-positive bacteria by reaction of the arginine-binding protein, thus affecting the arginine utilization [126].

4.5 Reuterin

Reuterin is a product by heterofermentative of *Lactobacillus reuteri*, species a member of microbiota of human and animal gastrointestinal tract. Reuterin presented a broad spectrum of antimicrobial activity against pathogens such as Gram-positive and Gram-negative bacteria, yeast, fungi and protozoa. The organisms have sensitive to reuterin such as *Salmonella, Shigella, Clostridium, Staphylococcus, Listeria, Candida*, and *Trypanosoma* [127].

4.6 Bacteriocins

Bacteriocins are proteinaceous compounds produced by bacteria strains in order to inhibit the growth of other bacteria. Bacteriocins groups are classified base on molecular weight differences. Class I- bacteriocins are small peptides (<5 kDa), Class II- small hydrophobic bacteriocins are heat-stable peptides (<13 kDa), Class III- large bacteriocins are heat-labile proteins (>30 kDa) and Class IV- complex bacteriocins are proteins with lipid and/or carbohydrate moieties [128]. The activity spectrum of bacteriocins can be narrow and confined to inhibition of closely related species, or it can be relatively broad and include many different bacterial species.

5. Methods for evaluation of antimicrobial activity

5.1 The agar diffusion method

The agar diffusion method was first used by Fleming in 1924. The method used for detection of antimicrobial activity and has long been widely used for evaluation of antimicrobial activity, especially for biologically derived compounds. It including agar well diffusion assay and disc assay. In this test, an antimicrobial compound is applied to an agar plate on a paper disc or in a well. The compound diffuses into agar resulting in a concentration gradient that is inversely proportional to the distance from the disc or well. The size of the inhibition zone able measured of degree around the disc or well. The results of the test are generally qualitative [129]. The method requires that the indicator organisms must grow rapidly, uniformly, and aerobically. Since highly hydrophobic antimicrobial compounds cannot diffuse in agar, they are not suitable for tests by this method [130]. The method with modified for used testing antimicrobial activity based on the agar diffusion method such as agar spot method [131] and spot-on-lawn method [132].

5.2 The agar and broth dilution methods

Agar and broth dilution methods are quantitative methods for suit microorganisms with variable growth rate and for anaerobic, microaerophilic microorganisms. The results are exhibited as MIC, which is the lowest concentration of an antimicrobial that prevents growth of a microorganism after a specific incubation period. In this test, serial dilution of antimicrobial and add a single concentration to culture tube (nonselective broth) or plate (melted agar medium), which is then inoculated with test organisms and incubated. The MIC is defined as the lowest concentration at which no growth occurs (absence of turbidity) in a medium following incubation [129]. The broth dilution assay has been used for the determination of the antimicrobial activity of reuterin produced by *Lb. reuteri*, and the activity of reuterin was exhibited as MIC values or as the maximum dilutions of the reuterin fraction[127].

5.3 The automated turbidometric assay

A turbidometric assay used for determines the effect of a compound on the growth or death kinetics of a microorganism. The assay is based on automated systems. It result shown information concerning the effect of an antimicrobial that may cause a delayed lag phase or reduced growth rate at concentrations below the MIC. Since the bacterial growth is monitored by measuring the turbidity of the broth medium, the method demands that the instrument be highly sensitive [133].

CHAPTER III

MATERIAL AND METHODS

1. Human subjects and sample collection

Breast milk were collected from healthy lactating Thai women who brought infants to receive vaccination at well baby clinic, 9th floor Por-Por-Ror building, King Chulalongkorn Memorial Hospital. Participated volunteers were enrolled according to following criteria (i) healthy women without present or past underlying condition (ii) aged 18-40 years (iii) lactating at range 15 days to 60 days and (iv) never received antibiotics during pregnancy and at least 1 month before sample collection. The sample calculated from the formula n = Z_{α}^{2}P (1-P) / $e^{2}$where Z_{α}= 1.96, P = 0.07, e = 0.05) was found to be 99.99. In this study, a total of 102 milk samples were then collected from volunteers, All volunteers were gave written informed consent to the protocol, which were approved by Ethical Committee of Faculty of Medicine, Chulalongkorn University. The participants provide samples of breast milk and, breast skin swabs. Nipple and mammary were first cleaned with sterile water and skin sampling was performed using sterile cotton swabs to rub around area of the outer quarter of breast and placed into a sterile tubes containing 0.15% peptone water. The milk samples were collected in sterile tube by manual expression using sterile gloves. The milk samples and skin swabs were kept in an icebox and transported to the laboratory within 3 h and immediately cultured on appropriate media.

2. Bacterial cultivation

Three culture media were used: (a) MRS medium (de Man, Rogosa and Sharpe) for isolation of lactobacilli. (b) MC medium (Modified Columbia, with 0.03 g/l bromocresol purple) as described by Beerens [68] for isolation of bifidobacteria. (c) M17 medium for isolation of streptococci. Breast milk samples of 1 ml were diluted in 9 ml buffered peptone water and ten-fold serial diluted to 10^{-2}-10^{-3} Diluted sample of 100 µl were spreaded onto MRS, MC, and M17 medium. Plates of MRS and MC media were incubated under anaerobic condition for 48-72 h. at 37 °C in an anaerobic chamber. Plates of M17 medium were incubated under aerobic condition for 24-48 h. at 37 °C. The remaining samples were kept at -80 °C for experimental use. Skin swabs were plated on the above media and incubated with plates of milk samples.

3. Selection of *Lactobacillus, Bifidobacterium,* and *Streptococcus* isolates

After incubation, colonies developed on MRS, MC, and M17 media were selected according to different morphologies. Colonies of each morphotype were tested for catalase activity and catalase-negative colonies were Gram-stained and microscopically examined. Subcultured were performed to obtain isolated colonies on appropriate media. Catalase-negative, Gram-positive rods or coccobacilli were tentatively considered *Lactobacillus*-like bacteria. Catalase-negative, Gram-positive bifid-shaped rods were tentatively considered *Bifidobacterium*-like bacteria and catalase- negative, Gram-positive cocci in chain were tentatively considered *Streptococcu*s-like bacteria. Isolates of suspected lactobacilli, bifidobacteria, and streptococci were kept in MRS, brain heart infusion broth (BHB), and M17 with 20% glycerol respectively, and stored in frozen cultures at -80 °C for experimental use.

4. Genotypic identification of *Lactobacillus, Bifidibacterium,* and *Streptococcus* isolates

Bacterial colonies were first tested with genus-specific primers. Bacterial DNAs were extracted from 2-3 colonies as follow: colonies were picked and put in to an eppendorf tube. After 200 µl sterile water was added, the suspension was mixed and spun down to remove water and resuspended with 180 µl sterile water. Solution of 20 µl 10X digestion buffer (5% tween 20 and 10 mg/ml proteinase K in 0.2 M Tris pH 8.3) was added and incubated at 60 °C for 1 h. After inactivation of proteinase K at 100 °C for 15 min, the suspension was centrifuged at 13,000 rpm for 5 min.

Supernatant was collected for amplification and identification of *Lactobacillus, Bifidobacterium,* and *Streptococcus* using 16S rRNA genes sequencing.

Lactobacillus genus-specific primers L159F (5'-GGA AAC AG(A/G) TGC TAA TAC CG-3') and L677R (5'-CAC CGC TAC ACA TGG AG-3'),[134] were used. The 25-μl reaction mixture contains 12.5 μl of Hot start master mix (GE Healthcare illustra, UK), 10 pmol of each primer, 5 μl DNA template and 2.5 μl H_2O. Amplification was performed: 95°C for 5 min; 35 cycles of 95 °C for 30 s, 57 °C for 1 min, and 72 °C for 1 min and a final extension of 72 °C for 5 min.

Bifidobacterium genus-specific primers Bif164F (5'-GGG TGG TAA TGC CGG ATG-3') and Bif601R (5'-TAA GCG ATG GAC TTT CAC ACC-3'),[135] were used. The 25-μl reaction mixture contains 12.5 μl of Hot start master mix (GE Healthcare illustra, UK), 10 pmol of each primer, 5 μl DNA template and 2.5 μl H_2O. Amplification was performed: 95 °C for 5 min; 35 cycles of 95 °C for 1 min, 59 °C for 1 min, and 72 °C for 1 min and a final extension of 72 °C for 10 min.

Streptococcus genus-specific primer Tuf-Strp-1 (5'- GAA GAA TTG CTT GAA TTG GTT GAA-3') and Tuf-Strep-R (5'- GGA CGG TAG TTG TTG AAG AAT GG-3') [136] were used. The 25-μl reaction mixture contains 12.5 μl of Hot start master mix (GE Healthcare illustra, UK), 10 pmol of each primer, 5 μl DNA template and 2.5 μl H_2O. Amplification was performed: 95 °C for 5 min; 35 cycles of 95 °C for 1 min, 56° C for 1 min, and 72 °C for 1 min and a final extension of 72 °C for 10 min.

Bacterial isolate which gave positive result with genus-specific primers, was subjected to DNA sequencing. The 16S rRNA gene sequences was amplified by PCR using the universal primer 16S-8F (5'-AGA GTT TGA TCY TGG YTY AG-3') and 16S-1541R (5'-AAG GAG GTG WTC CAR CC-3') [137] for genus *Lactobacillus*. The 50-μl reaction mixture contains 25 μl of Hot start master mix (GE Healthcare illustra, UK), 10 pmol primer, 5 μl DNA template and 15 μl H_2O. Amplification was performed: 95 °C for 5 min; 35 cycles of 95 °C for 30 s, 57 °C for 1 min, and 72 °C for 1 min and a final extension of 72 °C for 10 min. Genus *Bifidobacterium* was amplified using the universal primers lm26 (5'-GAT TCT GGC TCA GGA TGA ACG-3') and lm3 (5'-CGG GTG CTI CCC ACT TTC ATG-3') [138]. The 50-μl reaction mixture contains 25 μl of Hot start master mix (GE), 10 pmol of each primer, 5 μl DNA template and 15 μl H_2O. Amplification was performed: 95 °C for 5 min; 35

cycles of 94 °C for 1 min, 57 °C for 3 min, and 72 °C for 4 min and a final extension of 72 °C for 10 min. Genus *Streptococcus* was amplified using universal primers forward primer (5'-AGA GTT TGA TCC TGG CTC AG-3') and U926 (5'-CCG TCA ATT CCT TTR AGT TT-3') [139]. The 50-µl reaction mixture contains 25 µl of Hot start master mix (GE Healthcare illustra, UK), 10 pmol of each primer, 5 µl DNA template and 15 μl H_2O. Amplification was performed: 95 °C for 5 min; 35 cycles of 95 °C for 1 min, 56° C for 1 min, and 72 °C for 1 min and a final extension of 72 °C for 10 min.

PCR product was individually purified by using QIAquick PCR purification kit (Qiagen Inc., USA). Sequencing will be performed by using 10 ng purified PCR product with the same primer as in PCR amplification by the dideoxynucleotide chain termination method at the 1 st BASE Sequencing, Shan Alan, Malasia (http://www.base-asia.com). The nucleotide sequence will be analysed using the sequence match program of Ribosomal Database Project II (RDP-II; http://rdp.cme.msu.edu) and GenBank DNA database search (www.ncbi.nlm.nih.gov/BLAST). The closest relative of the partial 16S rRNA gene sequences was evaluated. The identities of the isolates were determined on the basis of the highest score.

5. Detection DNA of lactobacilli, bifidobacteria and streptococci by PCR method

DNA was isolated from 100 samples of breast milk stored at -80 °C by using QIAamp DNA stool minikit (Qiagen, Hilden, Germany). One milliliter of breast milk samples were centrifuged for 20 min at 6,000 rpm. After the supernatant was removed, used pellets and added a bead-beading with 0.3 g of 0.1 mm zirconium beads and 1.4 ml of ASL buffer (Qiagen) and mixed by vortex. The suspension was incubated at 95°C for 5 min and centrifuged, then supernatant was transferred to clean vial and an InhibitEX Tablet (Qiagen) was added. After centrifugation the supernatant was transferred to QIAamp spin columns (Qiagen) and made following the manufacturer’s instruction. DNA eluted in 200 µl of buffer AE (provided in the kit), and the extracted-purified DNA were stored at -20 °C. DNA targets were amplified by PCR using genus-specific primers L159F (5'-GGA AAC AG(A/G) TGC TAA

TAC CG-3') and L677R (5'-CAC CGC TAC ACA TGG AG-3') [134] for *Lactobacillus*. Bif164F (5’-GGG TGG TAA TGC CGG ATG-3’) and Bif601R (5'-TAA GCG ATG GAC TTT CAC ACC-3') [135] for *Bifidobacterium* and Tuf-Strep-1 (5'- GAA GAA TTG CTT GAA TTG GTT GAA-3') and Tuf-Strep-R (5'- GGA CGG TAG TTG TTG AAG AAT GG-3') [136] for *Streptococcus*. The PCR amplicons were detected with agarrose gel electrophoresis and stained with ethidium bromide.

6. Antagonistic activity assay

Antagonistic activities of *Lactobacillus*, *Bifidobacterium*, and *Streptococcus* against various bacterial pathogens were performed by agar spot method as previously described by Spinler *et al*[11]. Enterotoxigenic *E. coli* (ETEC) DMST 20970, enteroinvasive *E .coli* (EIEC) DMST 20971, enteropathogenic *E. coli* (EPEC) DMST 20972, enterohemorrhagic *E .coli* (EHEC) DMST 20973, *Salmonella* Typhimurium ATCC 13311, *Shigella flexneri* DMST 4423, *Vibrio cholerae* non O1 DMST 2873, *Helicobacter pylori* ATCC 43504 and Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300 were selected bacterial pathogen to be tested. All target bacteria were grown on appropriate media and condition for experimental use.

Lactobacillus and *Bifidobacterium* were precultivated on MRS and MC, respectively for 48-72 h. in an anaerobic condition (the AnaeroPack system, Mitsubishi Gas Chemical, H_2: 5%, CO_2: 10%, N_2: 85%). *Streptococcus* was precultivated on M17 agar in an aerobic condition for 24-48 h. They were subcultured on MRS, BHI and M17 broth (media for lactobacilli, bifidobacteria and streptococci, respectively) twice in a 96-well plate. Forty-eight hour culture of *Lactobacillus, Bifidobacterium,* and 24 h. of *Streptococcus* were spotted by frogger (Dan-Kar Corp, MA, USA) onto the surface of BHI agar in a 140-mm plate and incubated in anaerobic condition at 37 °C for 48 h., except plates of *Streptococcus* incubated in aerobic condition at 37 °C for 24 h. Twenty milliliters of tryptic soft agar (agar 7.5 g/l) containing target bacterial pathogens at concentration about 1×10^7CFU/ml (1×10^9CFU/ml of *Helicobacter pylori*) were overlain on plate of *Lactobacillus, Bifidobacterium,* and *Streptococcus* developed spots. Each plate was incubated under appropriate condition depending on each target pathogen. Inhibition zones were measured and a clear zone of 1-2 mm was scored as weak inhibitory activity, 3-4 mm as strong inhibitory activity and an opaque zone of inhibition <1 mm as microcolonies (M).

CHAPTER IV

RESULTS

1. Cultivation and presumptive identification of *Lactobacillus, Bifidobacterium* and *Streptocococus* from breast milk

One hundred and two milk samples and skin swabs were collected from Thai healthy mothers. These samples were cultured in MRS, MC and M17 agar for the isolation of *Lactobacillus, Bifidobacterium* and *Streptococcus,* respectively. The colonies of bacterial isolates from breast milk on MRS agar were small to medium, circular and convex, white or yellow turbid or transparent, whereas those on MC agar were small to medium, circular and convex or flat, white or yellow and transparent. On M17 agar, the colonies were medium to large, circular and convex or flat and white turbid. Colony morphologies of these bacteria on MRS, MC and M17 agar were shown in Figure 2. The colonies of bacterial isolates from skin swabs grown on MRS were small to medium, circular and convex or flat, white or yellow turbid or transparent (Figure 3 A). On MC agar, the colonies were small to medium, circular and convex or flat, white or yellow or green and turbid or transparent (Figure 3 B) and on M17 agar the colonies were medium to large, circular and convex or flat, white or grey and turbid or transparent (Figure 3 C).

Bacterial colonies grown on each medium with different appearance were picked and tested for catalase enzyme. The catalase-negative ones were Gram- stained and examined microscopically. Isolates visualized as Gram-positive short or long rods or coccobacilli on MRS agar were suspected of *Lactobacillus* (Figure 4 A). Isolates with Gram- positive, bifid or polymorphic branched or irregular rods on MC or MRS agar were suspected of *Bifidobacterium* (Figure 4 B) *and* isolates on M17 agar or MRS suspected to be *Streptococcus* were Gram-positive, cocci in chain or single (Figure 4 C). Suspected colonies were re-streaked for single colony isolation on new media. A single pure colony was re-tested for catalase enzyme, Gram- stained and examined microscopically.

A total of 176 bacterial isolates were selected for further identification. They were 74, 62 and 40 suspected *Lactobacillus*, *Bifidobacterium* and *Streptococcus*, respectively. All bacterial isolates from skin swabs were catalase-positive. They were either Gram-positive cocci in cluster or single Gram-negative cocci or Gram-negative cocci in small cluster.

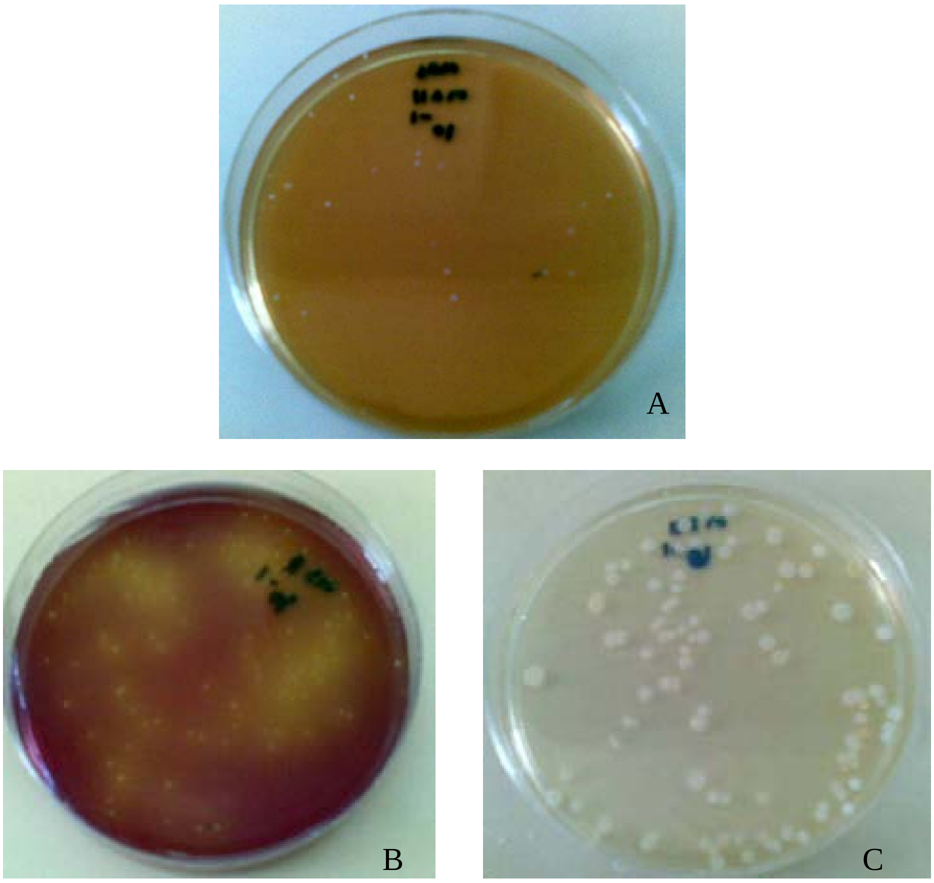


Figure 2. Colony growth from breast milk on different media, (A) MRS agar, (B) MC agar and (C) M17 agar.

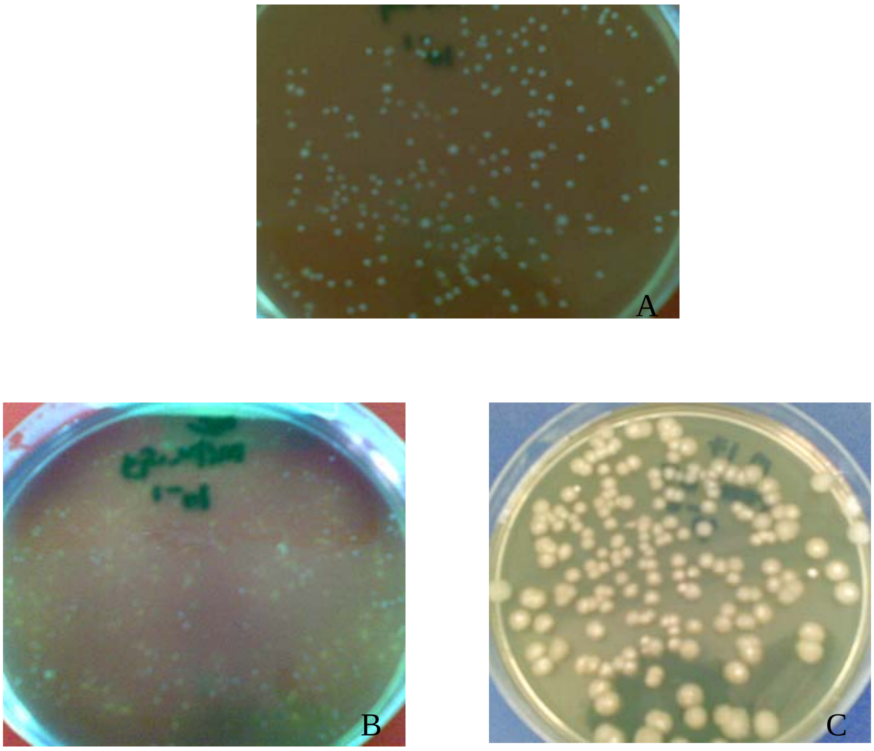


Figure 3. Colony growth from skin swabs on different media, (A) MRS agar, (B) MC agar and (C) M17 agar.

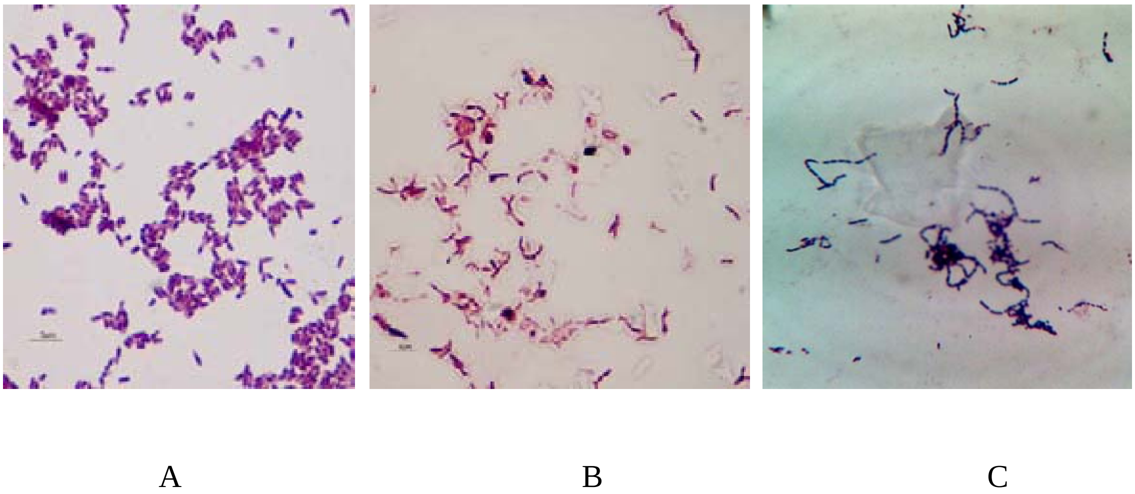


Figure 4. Cell morphology of bacteria isolated from breast milk, (A) Gram-positive short rods suspected to be *Lactobacillus* (B) Gram- positive bifid or irregular rods suspected to be *Bifidobacterium* (C) Gram-positive cocci in chain suspected to be *Streptococcus*.

2. Genotypic identification of lactobacilli, bifidobacteria and streptococci

DNAs of all 176 isolates were amplified using genus-specific primers. These genus-specific primers were aligned with the 16S rRNA gene sequence of *Lactobacillus* spp., *Bifidobacterium* spp., and *Streptococcus* spp. with Multalin program and the result was shown in Figures 5-7 Out of 74 suspected *Lactobacillus* isolates, 53 (71.62 %) were positive for *Lactobacillus*. Out of 62 suspected *Bifidobacterium* isolates, 45 (72.58%) were positive for *Bifidobacterium*. Out of 40 suspected *Streptococcus* isolates, 26 (65%) were positive for *Streptococcus*. Isolates with positive results from genus-specific amplification were then amplified using universal primers. These universal primers were aligned with the 16S rRNA gene sequence of *Lactobacillus* spp., *Bifidobacterium* spp., and *Streptococcus* spp. with Multalin program and the result was shown in Figures 8-10.

The amplification products were sequenced and analysed with NCBI and RDP II database. Forty out of 53 isolates (75.47%) were identified as *Lactobacillus* spp. such as *L. gasseri* (6 isolates), *L. salivarius* (16 isolates), *L. fermentum* (5 isolates), *L. mucosae* (5 isolates), *L. rhamnosus* (3 isolates), *L. casei* (3 isolates), *L. plantarum* (1 isolate) and *L. oris* (1 isolate) as shown in Table 6. These *Lactobacillus* isolates were recovered from 37 (36.27%) milk samples. Thirty-three out of 45 isolates (73.33%) were identified as *Bifidobacterium* spp. such as *B. longum* (8 isolates), *B. breve* (7 isolates), *B. psedocatenulatum* (5 isolates), *B. dentium* (8 isolates) and *B. bifidum* (5 isolates) as shown in Table 7. These *Bifidobacterium* isolates were recovered from 31 (30.39%) milk samples. All 26 isolates which were positive by *Streptococcus*-specific PCR were identified as *Streptococcus* spp. such as *S. salivarius* (13 isolates), *S. lactarius* (4 isolates), *Streptococcus* sp. (4 isolates), *Streptococcus mitis* (3 isolates) and *Streptococcus parasanguis* (2 isolates) as shown in Table 8. These *Streptococcus* isolates were recovered from 17 (16.67%) milk samples. The summary of bacterial isolates recovered from breast milk was shown in Tables 9-10.

Thirteen isolates positive with *Lactobacillus*-specific amplification were identified to be *Staphylococcus aureus* (5 isolates), *Staphylococcus epidermidis* (4 isolates) and uncultured bacteria (4 isolates). Twelve isolates positive with *Bifidobacterium*-specific amplification were identified to be *Actinomyces radicidentis*

(1 isolate) and uncultured bacteria (11 isolates). The summary of other bacterial species found in breast milk was shown in Table 11.

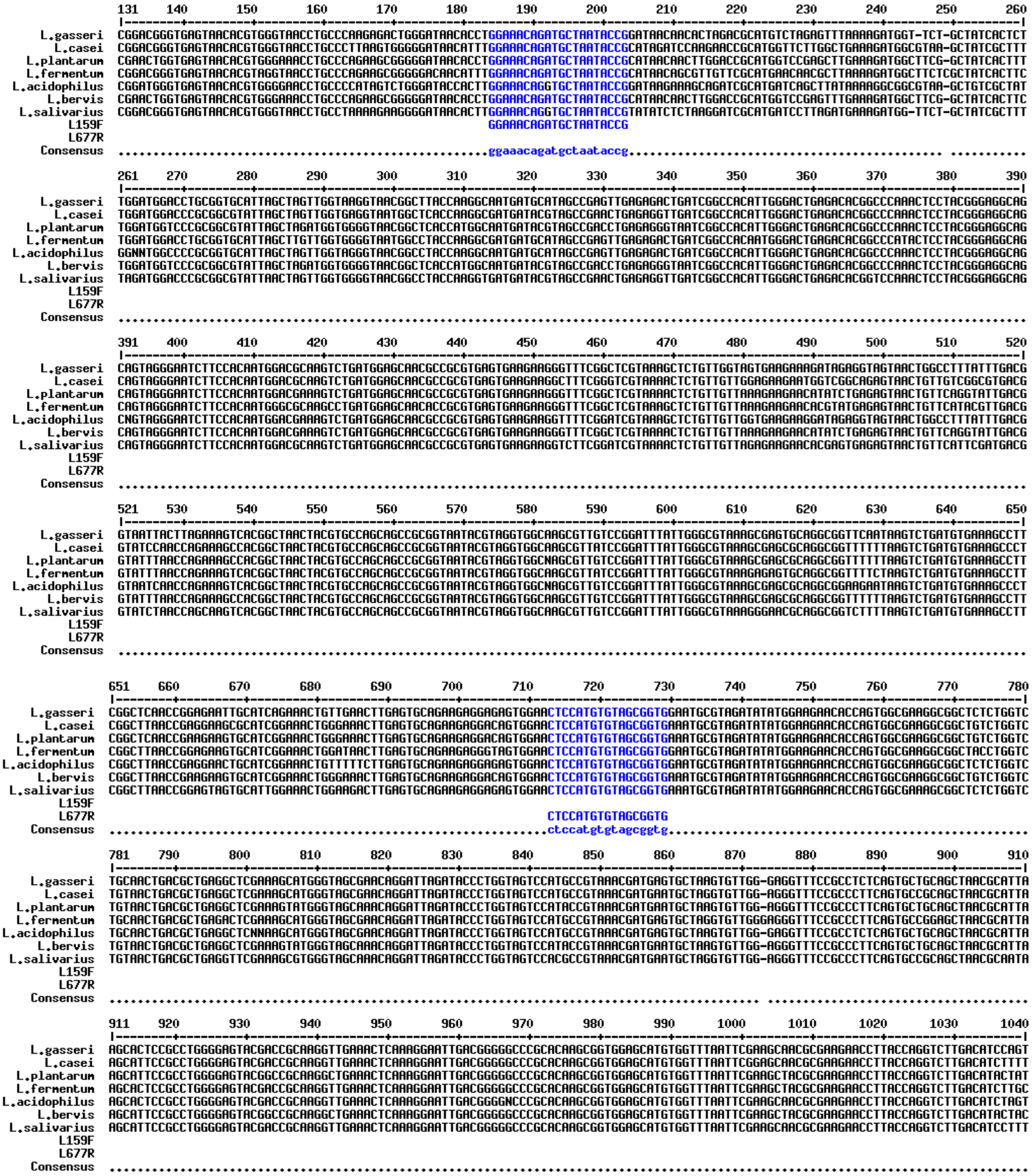
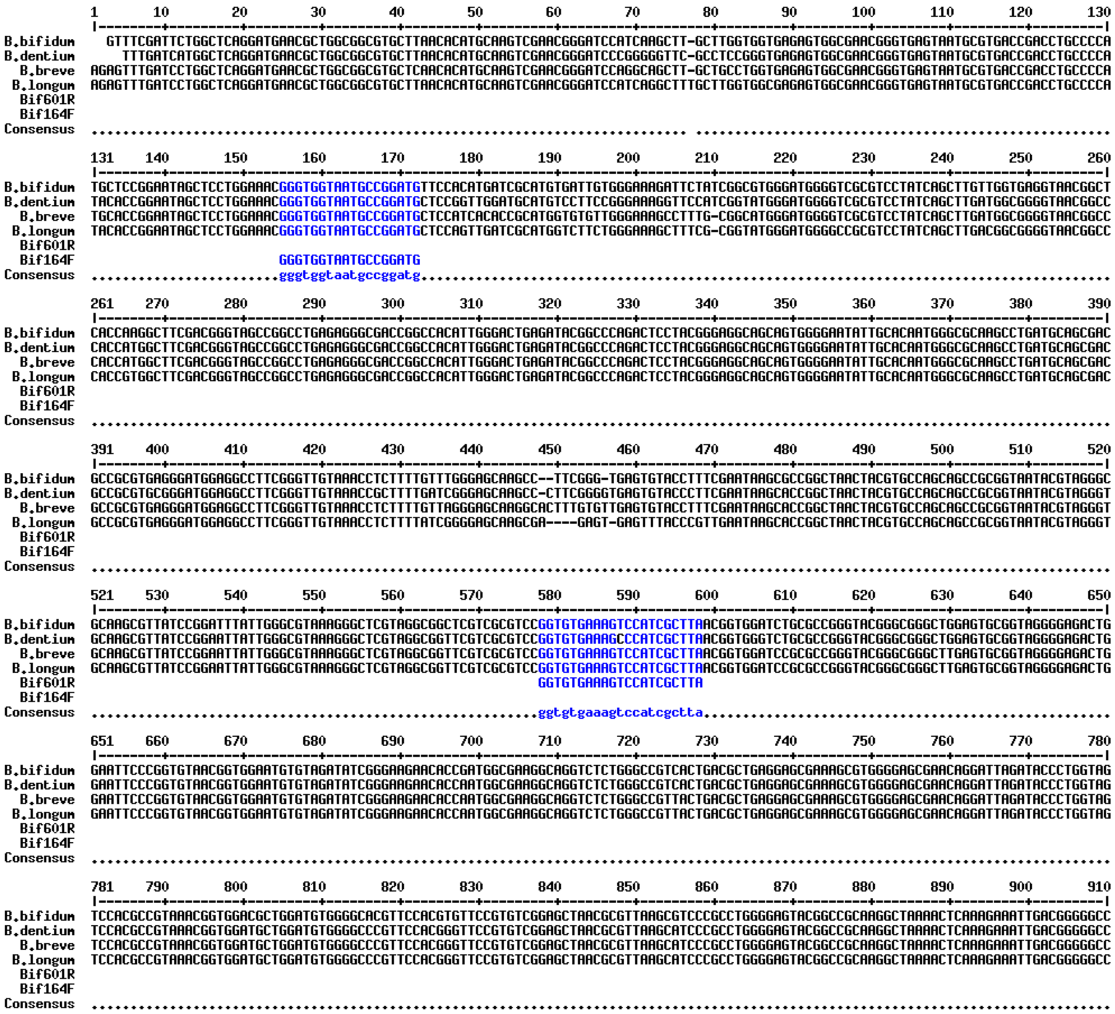


Figure 5. The alignment of genus-specific primer L159F and L677R with 16S rRNA gene sequence of *Lactobacillus* spp.



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          1       10        20        30        40        50        60        70        80        90       100       110       120       130
          |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
B.bifidum   GTTTCGATTCTGGCTCAGGATGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGGGATCCATCAAGCTT-GCTTGGTGGTGAGAGTGGCGAACGGGTGAGTAATGCGTGACCGACCTGCCCCA
B.dentium     TTTGATCATGGCTCAGGATGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGGGATCCCGGGGTTC-GCCTCCGGGTGAGAGTGGCGAACGGGTGAGTAATGCGTGACCGACCTGCCCCA
  B.breve AGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCTCAACACATGCAAGTCGAACGGGATCCAGGCAGCTT-GCTGCCTGGTGAGAGTGGCGAACGGGTGAGTAATGCGTGACCGACCTGCCCCA
 B.longum AGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGGGATCCATCAGGCTTTGCTTGGTGGCGAGAGTGGCGAACGGGTGAGTAATGCGTGACCGACCTGCCCCA
  Bif601R
  Bif164F
Consensus ............................................................................ .....................................................

          131    140       150       160       170       180       190       200       210       220       230       240       250       260
          |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
B.bifidum TGCTCCGGAATAGCTCCTGGAAACGGGTGGTAATGCCGGATGTTCCACATGATCGCATGTGATTGTGGGAAAGATTCTATCGGCGTGGGATGGGGTCGCGTCCTATCAGCTTGTTGGTGAGGTAACGGCT
B.dentium TACACCGGAATAGCTCCTGGAAACGGGTGGTAATGCCGGATGCTCCGGTTGGATGCATGTCCTTCCGGGAAAGGTTCCATCGGTATGGGATGGGGTCGCGTCCTATCAGCTTGATGGCGGGGTAACGGCC
  B.breve TGCACCGGAATAGCTCCTGGAAACGGGTGGTAATGCCGGATGCTCCATCACACCGCATGGTGTGTTGGGAAAGCCTTTG-CGGCATGGGATGGGGTCGCGTCCTATCAGCTTGATGGCGGGGTAACGGCC
 B.longum TACACCGGAATAGCTCCTGGAAACGGGTGGTAATGCCGGATGCTCCAGTTGATCGCATGGTCTTCTGGGAAAGCTTTCG-CGGTATGGGATGGGGCCGCGTCCTATCAGCTTGACGGCGGGGTAACGGCC
  Bif601R
  Bif164F                         GGGTGGTAATGCCGGATG
Consensus ........................gggtggtaatgccggatg........................................................................................

          261    270       280       290       300       310       320       330       340       350       360       370       380       390
          |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
B.bifidum CACCAAGGCTTCGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACATTGGGACTGAGATACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGAC
B.dentium CACCATGGCTTCGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACATTGGGACTGAGATACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGAC
  B.breve CACCATGGCTTCGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACATTGGGACTGAGATACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGAC
 B.longum CACCGTGGCTTCGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACATTGGGACTGAGATACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGAC
  Bif601R
  Bif164F
Consensus ..................................................................................................................................

          391    400       410       420       430       440       450       460       470       480       490       500       510       520
          |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
B.bifidum GCCGCGTGAGGGATGGAGGCCTTCGGGTTGTAAACCTCTTTTGTTTGGGAGCAAGCC--TTCGGG-TGAGTGTACCTTTCGAATAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGC
B.dentium GCCGCGTGCGGGATGGAGGCCTTCGGGTTGTAAACCGCTTTTGATCGGGAGCAAGCC-CTTCGGGGTGAGTGTACCCTTCGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGT
  B.breve GCCGCGTGAGGGATGGAGGCCTTCGGGTTGTAAACCTCTTTTGTTAGGGAGCAAGGCACTTTGTGTTGAGTGTACCTTTCGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGT
 B.longum GCCGCGTGAGGGATGGAGGCCTTCGGGTTGTAAACCTCTTTTATCGGGGAGCAAGCGA----GAGT-GAGTTTACCCGTTGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGT
  Bif601R
  Bif164F
Consensus ..................................................................................................................................

          521    530       540       550       560       570       580       590       600       610       620       630       640       650
          |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
B.bifidum GCAAGCGTTATCCGGATTTATTGGGCGTAAAGGGCTCGTAGGCGGCTCGTCGCGTCCGGTGTGAAAGTCCATCGCTTAACGGTGGATCTGCGCCGGGTACGGGCGGGCTGGAGTGCGGTAGGGGAGACTG
B.dentium GCAAGCGTTATCCGGAATTATTGGGCGTAAAGGGCTCGTAGGCGGTTCGTCGCGTCCGGTGTGAAAGCCCATCGCTTAACGGTGGGTCTGCGCCGGGTACGGGCGGGCTGGAGTGCGGTAGGGGAGACTG
  B.breve GCAAGCGTTATCCGGAATTATTGGGCGTAAAGGGCTCGTAGGCGGTTCGTCGCGTCCGGTGTGAAAGTCCATCGCTTAACGGTGGATCCGCGCCGGGTACGGGCGGGCTTGAGTGCGGTAGGGGAGACTG
 B.longum GCAAGCGTTATCCGGAATTATTGGGCGTAAAGGGCTCGTAGGCGGTTCGTCGCGTCCGGTGTGAAAGTCCATCGCTTAACGGTGGATCCGCGCCGGGTACGGGCGGGCTTGAGTGCGGTAGGGGAGACTG
  Bif601R                                                          GGTGTGAAAGTCCATCGCTTA
  Bif164F
Consensus .........................................................ggtgtgaaagtccatcgctta....................................................

          651    660       670       680       690       700       710       720       730       740       750       760       770       780
          |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
B.bifidum GAATTCCCGGTGTAACGGTGGAATGTGTAGATATCGGGAAGAACACCGATGGCGAAGGCAGGTCTCTGGGCCGTCACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAG
B.dentium GAATTCCCGGTGTAACGGTGGAATGTGTAGATATCGGGAAGAACACCAATGGCGAAGGCAGGTCTCTGGGCCGTCACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAG
  B.breve GAATTCCCGGTGTAACGGTGGAATGTGTAGATATCGGGAAGAACACCAATGGCGAAGGCAGGTCTCTGGGCCGTTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAG
 B.longum GAATTCCCGGTGTAACGGTGGAATGTGTAGATATCGGGAAGAACACCAATGGCGAAGGCAGGTCTCTGGGCCGTTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAG
  Bif601R
  Bif164F
Consensus ..................................................................................................................................

          781    790       800       810       820       830       840       850       860       870       880       890       900       910
          |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
B.bifidum TCCACGCCGTAAACGGTGGACGCTGGATGTGGGGCACGTTCCACGTGTTCCGTGTCGGAGCTAACGCGTTAAGCGTCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGAAATTGACGGGGGCC
B.dentium TCCACGCCGTAAACGGTGGATGCTGGATGTGGGGCCCGTTCCACGGGTTCCGTGTCGGAGCTAACGCGTTAAGCATCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGAAATTGACGGGGGCC
  B.breve TCCACGCCGTAAACGGTGGATGCTGGATGTGGGGCCCGTTCCACGGGTTCCGTGTCGGAGCTAACGCGTTAAGCATCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGAAATTGACGGGGGCC
 B.longum TCCACGCCGTAAACGGTGGATGCTGGATGTGGGGCCCGTTCCACGGGTTCCGTGTCGGAGCTAACGCGTTAAGCATCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGAAATTGACGGGGGCC
  Bif601R
  Bif164F
Consensus ..................................................................................................................................
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Figure 6. The alignment of genus-specific primer Bif164F and Bif601R with 16S rRNA gene sequence of *Bifidobacterium* spp.

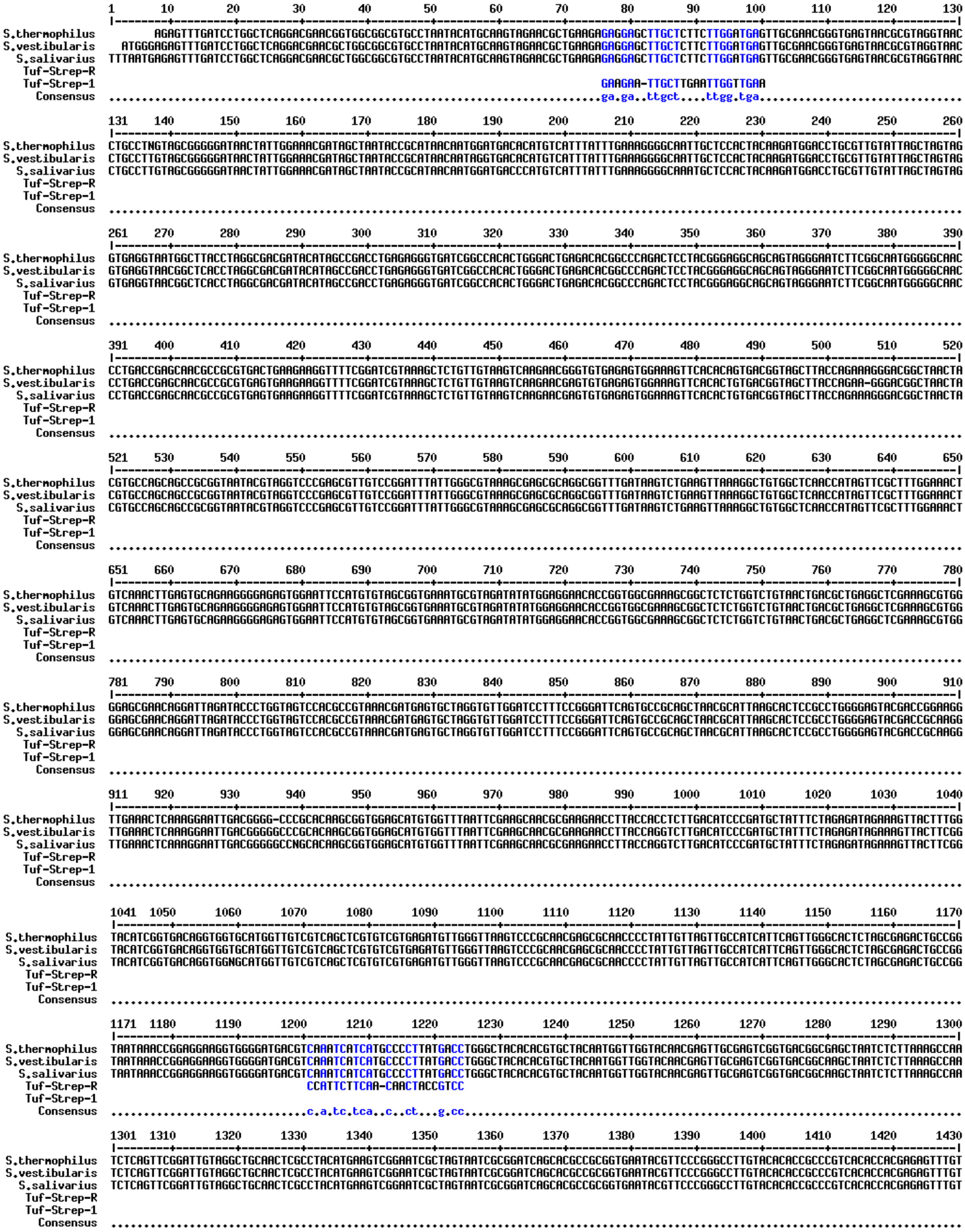
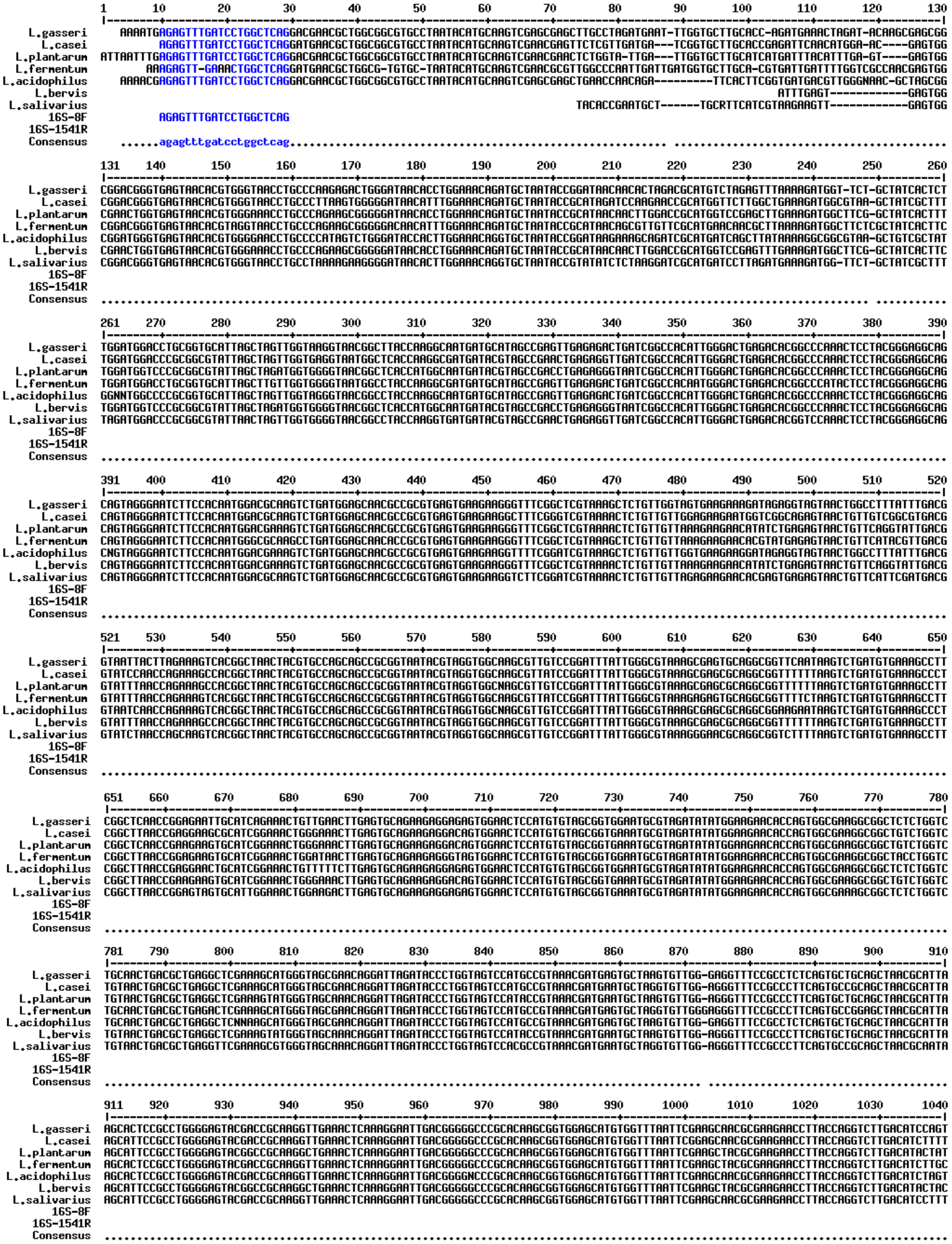


Figure 7. The alignment of genus-specific primer Tuf-Strep-1 and Tuf-Strep-R with 16S rRNA gene sequence of *Streptococcus* spp.



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              1       10        20        30        40        50        60        70        80        90       100       110       120       130
              |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
    L.gasseri    AAAATGAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGCTTGCCTAGATGAAT-TTGGTGCTTGCACC-AGATGAAACTAGAT-ACAAGCGAGCGG
      L.casei          AGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACGAGTTCTCGTTGATGA---TCGGTGCTTGCACCGAGATTCAACATGGA-AC----GAGTGG
  L.plantarum ATTAATTTGAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACGAACTCTGGTA-TTGA---TTGGTGCTTGCATCATGATTTACATTTGA-GT----GAGTGG
  L.fermentum        AAAGAGTT-GAAACTGGCTCAGGATGAACGCTGGCG-TGTGC-TAATACATGCAAGTCGAACGCGTTGGCCCAATTGATTGATGGTGCTTGCA-CGTGATTGATTTTGGTCGCCAACGAGTGG
L.acidophilus    AAAACGAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGCTGAACCAACAGA---------TTCACTTCGGTGATGACGTTGGGNAAC-GCTAGCGG
     L.bervis                                                                                                         ATTTGAGT------------GAGTGG
  L.salivarius                                                                          TACACCGAATGCT------TGCRTTCATCGTAAGAAGTT------------GAGTGG
       16S-8F          AGAGTTTGATCCTGGCTCAG
    16S-1541R
    Consensus    ......agagtttgatcctggctcag.......................................................... ..........................................

              131    140       150       160       170       180       190       200       210       220       230       240       250       260
              |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
    L.gasseri CGGACGGGTGAGTAACACGTGGGTAACCTGCCCAAGAGACTGGGATAACACCTGGAAACAGATGCTAATACCGGATAACAACACTAGACGCATGTCTAGAGTTTAAAAGATGGT-TCT-GCTATCACTCT
      L.casei CGGACGGGTGAGTAACACGTGGGTAACCTGCCCTTAAGTGGGGGATAACATTTGGAAACAGATGCTAATACCGCATAGATCCAAGAACCGCATGGTTCTTGGCTGAAAGATGGCGTAA-GCTATCGCTTT
  L.plantarum CGAACTGGTGAGTAACACGTGGGAAACCTGCCCAGAAGCGGGGGATAACACCTGGAAACAGATGCTAATACCGCATAACAACTTGGACCGCATGGTCCGAGCTTGAAAGATGGCTTCG-GCTATCACTTT
  L.fermentum CGGACGGGTGAGTAACACGTAGGTAACCTGCCCAGAAGCGGGGGACAACATTTGGAAACAGATGCTAATACCGCATAACAGCGTTGTTCGCATGAACAACGCTTAAAAGATGGCTTCTCGCTATCACTTC
L.acidophilus CGGATGGGTGAGTAACACGTGGGGAACCTGCCCCATAGTCTGGGATACCACTTGGAAACAGGTGCTAATACCGGATAAGAAAGCAGATCGCATGATCAGCTTATAAAAGGCGGCGTAA-GCTGTCGCTAT
     L.bervis CGAACTGGTGAGTAACACGTGGGAAACCTGCCCAGAAGCGGGGGATAACACCTGGAAACAGATGCTAATACCGCATAACAACTTGGACCGCATGGTCCGAGTTTGAAAGATGGCTTCG-GCTATCACTTC
  L.salivarius CGGACGGGTGAGTAACACGTGGGTAACCTGCCTAAAAGAAGGGGATAACACTTGGAAACAGGTGCTAATACCGTATATCTCTAAGGATCGCATGATCCTTAGATGAAAGATGG-TTCT-GCTATCGCTTT
       16S-8F
    16S-1541R
    Consensus ...................................................................................................................... ...........

              261    270       280       290       300       310       320       330       340       350       360       370       380       390
              |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
    L.gasseri TGGATGGACCTGCGGTGCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCAATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAG
      L.casei TGGATGGACCCGCGGCGTATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGATGATACGTAGCCGAACTGAGAGGTTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAG
  L.plantarum TGGATGGTCCCGCGGCGTATTAGCTAGATGGTGGGGTAACGGCTCACCATGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAG
  L.fermentum TGGATGGACCTGCGGTGCATTAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACAATGGGACTGAGACACGGCCCATACTCCTACGGGAGGCAG
L.acidophilus GGNNTGGCCCCGCGGTGCATTAGCTAGTTGGTAGGGTAACGGCCTACCAAGGCAATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAG
     L.bervis TGGATGGTCCCGCGGCGTATTAGCTAGATGGTGGGGTAACGGCTCACCATGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAG
  L.salivarius TAGATGGACCCGCGGCGTATTAACTAGTTGGTGGGGTAACGGCCTACCAAGGTGATGATACGTAGCCGAACTGAGAGGTTGATCGGCCACATTGGGACTGAGACACGGTCCAAACTCCTACGGGAGGCAG
       16S-8F
    16S-1541R
    Consensus ..................................................................................................................................

              391    400       410       420       430       440       450       460       470       480       490       500       510       520
              |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
    L.gasseri CAGTAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAGCTCTGTTGGTAGTGAAGAAAGATAGAGGTAGTAACTGGCCTTTATTTGACG
      L.casei CAGTAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTGGAGAAGAATGGTCGGCAGAGTAACTGTTGTCGGCGTGACG
  L.plantarum CAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTAACTGTTCAGGTATTGACG
  L.fermentum CAGTAGGGAATCTTCCACAATGGGCGCAAGCCTGATGGAGCAACACCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAGCTCTGTTGTTAAAGAAGAACACGTATGAGAGTAACTGTTCATACGTTGACG
L.acidophilus CNGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTGGTGAAGAAGGATAGAGGTAGTAACTGGCCTTTATTTGACG
     L.bervis CAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTAACTGTTCAGGTATTGACG
  L.salivarius CAGTAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTCTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACACGAGTGAGAGTAACTGTTCATTCGATGACG
       16S-8F
    16S-1541R
    Consensus ..................................................................................................................................

              521    530       540       550       560       570       580       590       600       610       620       630       640       650
              |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
    L.gasseri GTAATTACTTAGAAAGTCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGTGCAGGCGGTTCAATAAGTCTGATGTGAAAGCCTT
      L.casei GTATCCAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTTTAAGTCTGATGTGAAAGCCCT
  L.plantarum GTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCNAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTTTAAGTCTGATGTGAAAGCCTT
  L.fermentum GTATTTAACCAGAAAGTCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTTATTGGGCGTAAAGAGAGTGCAGGCGGTTTTCTAAGTCTGATGTGAAAGCCTT
L.acidophilus GTAATCAACCAGAAAGTCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCNAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGAAGAATAAGTCTGATGTGAAAGCCCT
     L.bervis GTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTTTAAGTCTGATGTGAAAGCCTT
  L.salivarius GTATCTAACCAGCAAGTCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGGGAACGCAGGCGGTCTTTTAAGTCTGATGTGAAAGCCTT
       16S-8F
    16S-1541R
    Consensus ..................................................................................................................................

              651    660       670       680       690       700       710       720       730       740       750       760       770       780
              |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
    L.gasseri CGGCTCAACCGGAGAATTGCATCAGAAACTGTTGAACTTGAGTGCAGAAGAGGAGAGTGGAACTCCATGTGTAGCGGTGGAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGTC
      L.casei CGGCTTAACCGAGGAAGCGCATCGGAAACTGGGAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTC
  L.plantarum CGGCTCAACCGAAGAAGTGCATCGGAAACTGGGAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTC
  L.fermentum CGGCTTAACCGGAGAAGTGCATCGGAAACTGGATAACTTGAGTGCAGAAGAGGGTAGTGGAACTCCATGTGTAGCGGTGGAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTACCTGGTC
L.acidophilus CGGCTTAACCGAGGAACTGCATCGGAAACTGTTTTTCTTGAGTGCAGAAGAGGAGAGTGGAACTCCATGTGTAGCGGTGGAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGTC
     L.bervis CGGCTTAACCGAAGAAGTGCATCGGAAACTGGGAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTC
  L.salivarius CGGCTTAACCGGAGTAGTGCATTGGAAACTGGAAGACTTGAGTGCAGAAGAGGAGAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAAGCGGCTCTCTGGTC
       16S-8F
    16S-1541R
    Consensus ..................................................................................................................................

              781    790       800       810       820       830       840       850       860       870       880       890       900       910
              |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
    L.gasseri TGCAACTGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGAGTGCTAAGTGTTGG-GAGGTTTCCGCCTCTCAGTGCTGCAGCTAACGCATTA
      L.casei TGTAACTGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGAATGCTAGGTGTTGG-AGGGTTTCCGCCCTTCAGTGCCGCAGCTAACGCATTA
  L.plantarum TGTAACTGACGCTGAGGCTCGAAAGTATGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCATACCGTAAACGATGAATGCTAAGTGTTGG-AGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTA
  L.fermentum TGCAACTGACGCTGAGACTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGAGTGCTAGGTGTTGGGAGGGTTTCCGCCCTTCAGTGCCGGAGCTAACGCATTA
L.acidophilus TGCAACTGACGCTGAGGCTCNNAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGAGTGCTAAGTGTTGG-GAGGTTTCCGCCTCTCAGTGCTGCAGCTAACGCATTA
     L.bervis TGTAACTGACGCTGAGGCTCGAAAGTATGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCATACCGTAAACGATGAATGCTAAGTGTTGG-AGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTA
  L.salivarius TGTAACTGACGCTGAGGTTCGAAAGCGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATGCTAGGTGTTGG-AGGGTTTCCGCCCTTCAGTGCCGCAGCTAACGCAATA
       16S-8F
    16S-1541R
    Consensus ............................................................................................ .....................................

              911    920       930       940       950       960       970       980       990      1000      1010      1020      1030      1040
              |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
    L.gasseri AGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCAGT
      L.casei AGCATTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGGAGCAACGCGAAGAACCTTACCAGGTCTTGACATCTTTT
  L.plantarum AGCATTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTGACATACTAT
  L.fermentum AGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTGACATCTTGC
L.acidophilus AGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGNCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCTAGT
     L.bervis AGCATTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTGACATACTAC
  L.salivarius AGCATTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTTT
       16S-8F
    16S-1541R
    Consensus ..................................................................................................................................
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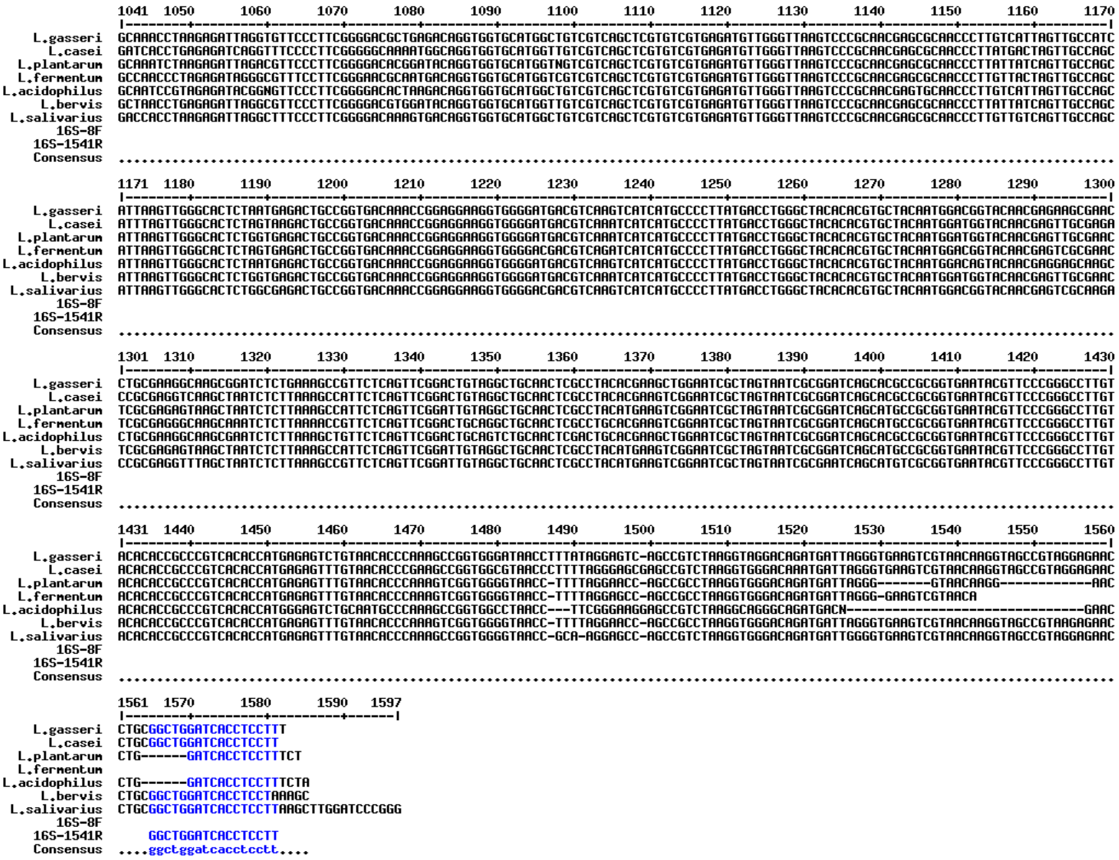

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              1041  1050      1060      1070      1080      1090      1100      1110      1120      1130      1140      1150      1160      1170
              |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
    L.gasseri GCAAACCTAAGAGATTAGGTGTTCCCTTCGGGGACGCTGAGACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCATTAGTTGCCATC
      L.casei GATCACCTGAGAGATCAGGTTTCCCCTTCGGGGGCAAAATGGCAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATGACTAGTTGCCAGC
  L.plantarum GCAAATCTAAGAGATTAGACGTTCCCTTCGGGGACACGGATACAGGTGGTGCATGGTNGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTATCAGTTGCCAGC
  L.fermentum GCCAACCCTAGAGATAGGGCGTTTCCTTCGGGAACGCAATGACAGGTGGTGCATGGTCGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTACTAGTTGCCAGC
L.acidophilus GCAATCCGTAGAGATACGGNGTTCCCTTCGGGGACACTAAGACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGTGCAACCCTTGTCATTAGTTGCCAGC
     L.bervis GCTAACCTGAGAGATTAGGCGTTCCCTTCGGGGACGTGGATACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTATCAGTTGCCAGC
 L.salivarius GACCACCTAAGAGATTAGGCTTTCCCTTCGGGGACAAAGTGACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTGTCAGTTGCCAGC
       16S-8F
    16S-1541R
    Consensus ..................................................................................................................................

              1171  1180      1190      1200      1210      1220      1230      1240      1250      1260      1270      1280      1290      1300
              |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
    L.gasseri ATTAAGTTGGGCACTCTAATGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAACGAGAAGCGAAC
      L.casei ATTTAGTTGGGCACTCTAGTAAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAACGAGTTGCGAGA
  L.plantarum ATTAAGTTGGGCACTCTGGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAACGAGTTGCGAAC
  L.fermentum ATTAAGTTGGGCACTCTAGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGACGACGTCAGATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAACGAGTCGCGAAC
L.acidophilus ATTAAGTTGGGCACTCTAATGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACAGTACAACGAGGAGCAAGC
     L.bervis ATTAAGTTGGGCACTCTGGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAACGAGTTGCGAAC
 L.salivarius ATTAAGTTGGGCACTCTGGCGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAACGAGTCGCAAGA
       16S-8F
    16S-1541R
    Consensus ..................................................................................................................................

              1301  1310      1320      1330      1340      1350      1360      1370      1380      1390      1400      1410      1420      1430
              |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
    L.gasseri CTGCGAAGGCAAGCGGATCTCTGAAAGCCGTTCTCAGTTCGGACTGTAGGCTGCAACTCGCCTACACGAAGCTGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGT
      L.casei CCGCGAGGTCAAGCTAATCTCTTAAAGCCATTCTCAGTTCGGACTGTAGGCTGCAACTCGCCTACACGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGT
  L.plantarum TCGCGAGAGTAAGCTAATCTCTTAAAGCCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGT
  L.fermentum TCGCGAGGGCAAGCAAATCTCTTAAAACCGTTCTCAGTTCGGACTGCAGGCTGCAACTCGCCTGCACGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGT
L.acidophilus CTGCGAAGGCAAGCGAATCTCTTAAAGCTGTTCTCAGTTCGGACTGCAGTCTGCAACTCGACTGCACGAAGCTGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGT
     L.bervis TCGCGAGAGTAAGCTAATCTCTTAAAGCCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGT
 L.salivarius CCGCGAGGTTTAGCTAATCTCTTAAAGCCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGAATCAGCATGTCGCGGTGAATACGTTCCCGGGCCTTGT
       16S-8F
    16S-1541R
    Consensus ..................................................................................................................................

              1431  1440      1450      1460      1470      1480      1490      1500      1510      1520      1530      1540      1550      1560
              |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
    L.gasseri ACACACCGCCCGTCACACCATGAGAGTCTGTAACACCCAAAGCCGGTGGGATAACCTTTATAGGAGTC-AGCCGTCTAAGGTAGGACAGATGATTAGGGTGAAGTCGTAACAAGGTAGCCGTAGGAGAAC
      L.casei ACACACCGCCCGTCACACCATGAGAGTTTGTAACACCCGAAGCCGGTGGCGTAACCCTTTTAGGGAGCGAGCCGTCTAAGGTGGGACAAATGATTAGGGTGAAGTCGTAACAAGGTAGCCGTAGGAGAAC
  L.plantarum ACACACCGCCCGTCACACCATGAGAGTTTGTAACACCCAAAGTCGGTGGGGTAACC-TTTTAGGAACC-AGCCGCCTAAGGTGGGACAGATGATTAGGG-------GTAACAAGG------------AAC
  L.fermentum ACACACCGCCCGTCACACCATGAGAGTTTGTAACACCCAAAGTCGGTGGGGTAACC-TTTTAGGAGCC-AGCCGCCTAAGGTGGGACAGATGATTAGGG-GAAGTCGTAACA
L.acidophilus ACACACCGCCCGTCACACCATGGGAGTCTGCAATGCCCAAAGCCGGTGGCCTAACC---TTCGGGAAGGAGCCGTCTAAGGCAGGGCAGATGACN-------------------------------GAAC
     L.bervis ACACACCGCCCGTCACACCATGAGAGTTTGTAACACCCAAAGTCGGTGGGGTAACC-TTTTAGGAACC-AGCCGCCTAAGGTGGGACAGATGATTAGGGTGAAGTCGTAACAAGGTAGCCGTAAGAGAAC
 L.salivarius ACACACCGCCCGTCACACCATGAGAGTTTGTAACACCCAAAGCCGGTGGGGTAACC-GCA-AGGAGCC-AGCCGTCTAAGGTGGGACAGATGATTGGGGTGAAGTCGTAACAAGGTAGCCGTAGGAGAAC
       16S-8F
    16S-1541R
    Consensus ..................................................................................................................................

              1561  1570      1580      1590   1597
              |--------+---------+---------+------|
    L.gasseri CTGCGGCTGGATCACCTCCTTT
      L.casei CTGCGGCTGGATCACCTCCTT
  L.plantarum CTG------GATCACCTCCTTTCT
  L.fermentum
L.acidophilus CTG------GATCACCTCCTTTCTA
     L.bervis CTGCGGCTGGATCACCTCCTAAAGC
 L.salivarius CTGCGGCTGGATCACCTCCTTAAGCTTGGATCCCGGG
       16S-8F
    16S-1541R     GGCTGGATCACCTCCTT
    Consensus ....ggctggatcacctcctt....
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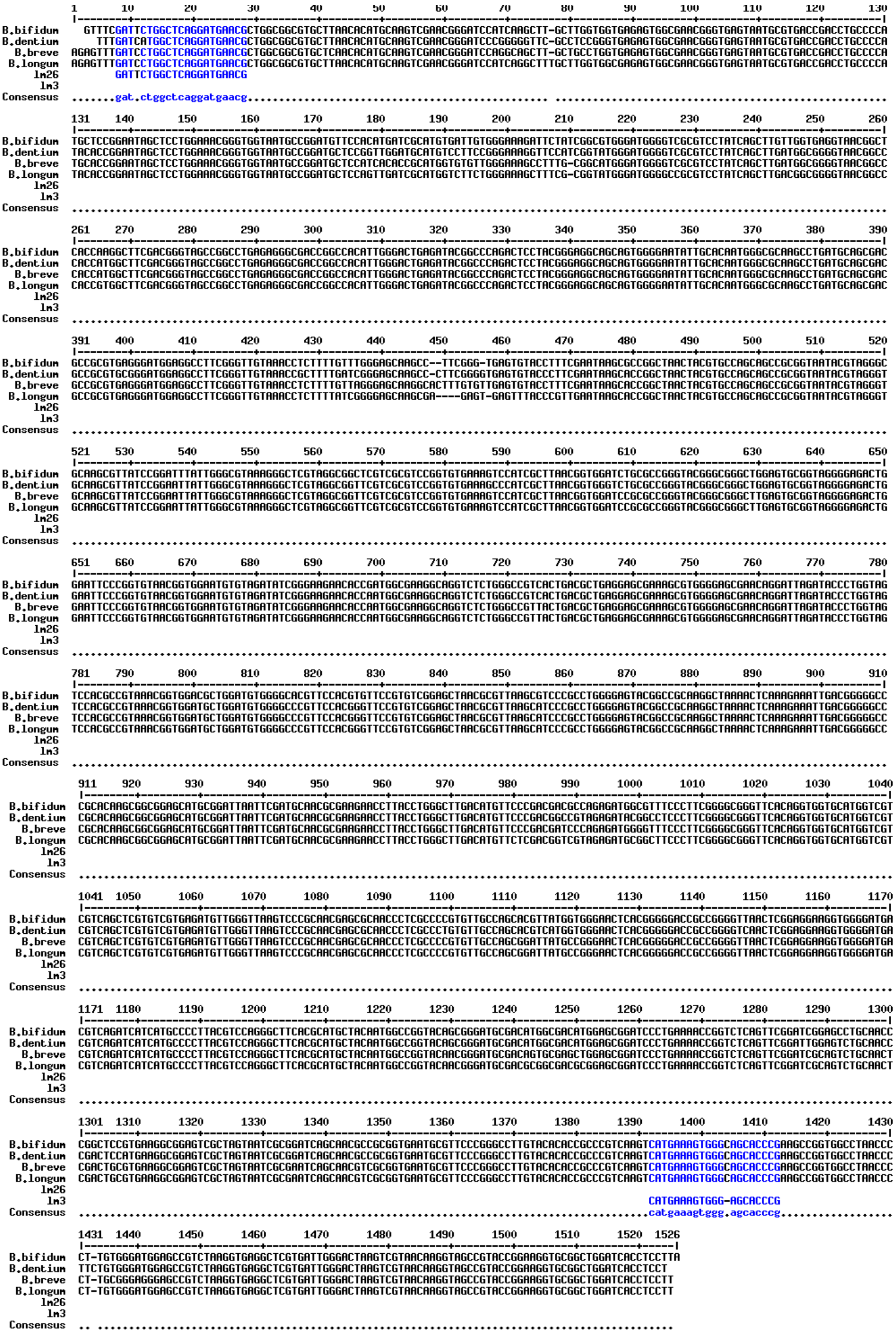
Figure 8. The alignment of universal primers 16S-8F and 16S-1541R with 16S rRNA gene sequence of *Lactobacillus* spp.



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          1       10        20        30        40        50        60        70        80        90       100       110       120       130
          |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
B.bifidum   GTTTCGATTCTGGCTCAGGATGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGGGATCCATCAAGCTT-GCTTGGTGGTGAGAGTGGCGAACGGGTGAGTAATGCGTGACCGACCTGCCCCA
B.dentium     TTTGATCATGGCTCAGGATGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGGGATCCCGGGGGTTC-GCCTCCGGGTGAGAGTGGCGAACGGGTGAGTAATGCGTGACCGACCTGCCCCA
  B.breve AGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCTCAACACATGCAAGTCGAACGGGATCCAGGCAGCTT-GCTGCCTGGTGAGAGTGGCGAACGGGTGAGTAATGCGTGACCGACCTGCCCCA
 B.longum AGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGGGATCCATCAGGCTTTGCTTGGTGGCGAGAGTGGCGAACGGGTGAGTAATGCGTGACCGACCTGCCCCA
     lm26        GATTCTGGCTCAGGATGAACG
      lm3
Consensus .......gat.ctggctcaggatgaacg................................................ .....................................................

          131    140       150       160       170       180       190       200       210       220       230       240       250       260
          |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
B.bifidum TGCTCCGGAATAGCTCCTGGAAACGGGTGGTAATGCCGGATGTTCCACATGATCGCATGTGATTGTGGGAAAGATTCTATCGGCGTGGGATGGGGTCGCGTCCTATCAGCTTGTTGGTGAGGTAACGGCT
B.dentium TACACCGGAATAGCTCCTGGAAACGGGTGGTAATGCCGGATGCTCCGGTTGGATGCATGTCCTTCCGGGAAAGGTTCCATCGGTATGGGATGGGGTCGCGTCCTATCAGCTTGATGGCGGGGTAACGGCC
  B.breve TGCACCGGAATAGCTCCTGGAAACGGGTGGTAATGCCGGATGCTCCATCACACCGCATGGTGTGTTGGGAAAGCCTTTG-CGGCATGGGATGGGGTCGCGTCCTATCAGCTTGATGGCGGGGTAACGGCC
 B.longum TACACCGGAATAGCTCCTGGAAACGGGTGGTAATGCCGGATGCTCCAGTTGATCGCATGGTCTTCTGGGAAAGCTTTCG-CGGTATGGGATGGGGCCGCGTCCTATCAGCTTGACGGCGGGGTAACGGCC
     lm26
      lm3
Consensus ..................................................................................................................................

          261    270       280       290       300       310       320       330       340       350       360       370       380       390
          |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
B.bifidum CACCAAGGCTTCGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACATTGGGACTGAGATACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGAC
B.dentium CACCATGGCTTCGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACATTGGGACTGAGATACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGAC
  B.breve CACCATGGCTTCGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACATTGGGACTGAGATACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGAC
 B.longum CACCGTGGCTTCGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACATTGGGACTGAGATACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGAC
     lm26
      lm3
Consensus ..................................................................................................................................

          391    400       410       420       430       440       450       460       470       480       490       500       510       520
          |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
B.bifidum GCCGCGTGAGGGATGGAGGCCTTCGGGTTGTAAACCTCTTTTGTTTGGGAGCAAGCC--TTCGGG-TGAGTGTACCTTTCGAATAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGC
B.dentium GCCGCGTGCGGGATGGAGGCCTTCGGGTTGTAAACCGCTTTTGATCGGGAGCAAGCC-CTTCGGGGTGAGTGTACCCTTCGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGT
  B.breve GCCGCGTGAGGGATGGAGGCCTTCGGGTTGTAAACCTCTTTTGTTAGGGAGCAAGGCACTTTGTGTTGAGTGTACCTTTCGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGT
 B.longum GCCGCGTGAGGGATGGAGGCCTTCGGGTTGTAAACCTCTTTTATCGGGGAGCAAGCGA----GAGT-GAGTTTACCCGTTGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGT
     lm26
      lm3
Consensus ..................................................................................................................................

          521    530       540       550       560       570       580       590       600       610       620       630       640       650
          |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
B.bifidum GCAAGCGTTATCCGGATTTATTGGGCGTAAAGGGCTCGTAGGCGGCTCGTCGCGTCCGGTGTGAAAGTCCATCGCTTAACGGTGGATCTGCGCCGGGTACGGGCGGGCTGGAGTGCGGTAGGGGAGACTG
B.dentium GCAAGCGTTATCCGGAATTATTGGGCGTAAAGGGCTCGTAGGCGGTTCGTCGCGTCCGGTGTGAAAGCCCATCGCTTAACGGTGGGTCTGCGCCGGGTACGGGCGGGCTGGAGTGCGGTAGGGGAGACTG
  B.breve GCAAGCGTTATCCGGAATTATTGGGCGTAAAGGGCTCGTAGGCGGTTCGTCGCGTCCGGTGTGAAAGTCCATCGCTTAACGGTGGATCCGCGCCGGGTACGGGCGGGCTTGAGTGCGGTAGGGGAGACTG
 B.longum GCAAGCGTTATCCGGAATTATTGGGCGTAAAGGGCTCGTAGGCGGTTCGTCGCGTCCGGTGTGAAAGTCCATCGCTTAACGGTGGATCCGCGCCGGGTACGGGCGGGCTTGAGTGCGGTAGGGGAGACTG
     lm26
      lm3
Consensus ..................................................................................................................................

          651    660       670       680       690       700       710       720       730       740       750       760       770       780
          |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
B.bifidum GAATTCCCGGTGTAACGGTGGAATGTGTAGATATCGGGAAGAACACCGATGGCGAAGGCAGGTCTCTGGGCCGTCACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAG
B.dentium GAATTCCCGGTGTAACGGTGGAATGTGTAGATATCGGGAAGAACACCAATGGCGAAGGCAGGTCTCTGGGCCGTCACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAG
  B.breve GAATTCCCGGTGTAACGGTGGAATGTGTAGATATCGGGAAGAACACCAATGGCGAAGGCAGGTCTCTGGGCCGTTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAG
 B.longum GAATTCCCGGTGTAACGGTGGAATGTGTAGATATCGGGAAGAACACCAATGGCGAAGGCAGGTCTCTGGGCCGTTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAG
     lm26
      lm3
Consensus ..................................................................................................................................

          781    790       800       810       820       830       840       850       860       870       880       890       900       910
          |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
B.bifidum TCCACGCCGTAAACGGTGGACGCTGGATGTGGGGCACGTTCCACGTGTTCCGTGTCGGAGCTAACGCGTTAAGCGTCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGAAATTGACGGGGGCC
B.dentium TCCACGCCGTAAACGGTGGATGCTGGATGTGGGGCCCGTTCCACGGGTTCCGTGTCGGAGCTAACGCGTTAAGCATCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGAAATTGACGGGGGCC
  B.breve TCCACGCCGTAAACGGTGGATGCTGGATGTGGGGCCCGTTCCACGGGTTCCGTGTCGGAGCTAACGCGTTAAGCATCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGAAATTGACGGGGGCC
 B.longum TCCACGCCGTAAACGGTGGATGCTGGATGTGGGGCCCGTTCCACGGGTTCCGTGTCGGAGCTAACGCGTTAAGCATCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGAAATTGACGGGGGCC
     lm26
      lm3
Consensus ..................................................................................................................................

          911    920       930       940       950       960       970       980       990      1000      1010      1020      1030      1040
          |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
B.bifidum CGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCTGGGCTTGACATGTTCCCGACGACGCCAGAGATGGCGTTTCCCTTCGGGGCGGGTTCACAGGTGGTGCATGGTCGT
B.dentium CGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCTGGGCTTGACATGTTCCCGACGGCCGTAGAGATACGGCCTCCCTTCGGGGCGGGTTCACAGGTGGTGCATGGTCGT
  B.breve CGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCTGGGCTTGACATGTTCCCGACGATCCCAGAGATGGGGTTTCCCTTCGGGGCGGGTTCACAGGTGGTGCATGGTCGT
 B.longum CGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCTGGGCTTGACATGTTCTCGACGGTCGTAGAGATGCGGCTTCCCTTCGGGGCGGGTTCACAGGTGGTGCATGGTCGT
     lm26
      lm3
Consensus ..................................................................................................................................

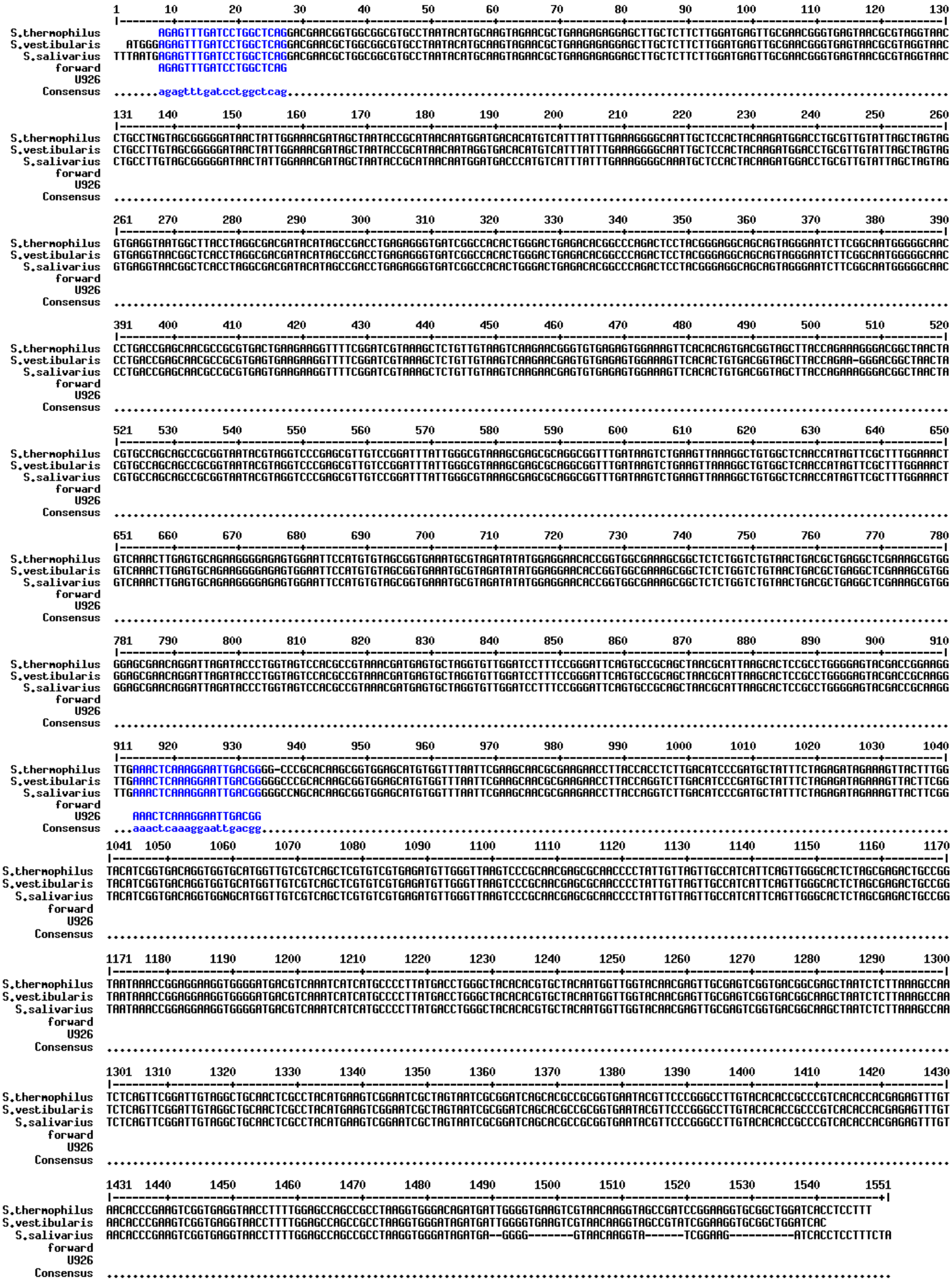
          1041  1050      1060      1070      1080      1090      1100      1110      1120      1130      1140      1150      1160      1170
          |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
B.bifidum CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGCCCCGTGTTGCCAGCACGTTATGGTGGGAACTCACGGGGGACCGCCGGGGTTAACTCGGAGGAAGGTGGGGATGA
B.dentium CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGCCCTGTGTTGCCAGCACGTCATGGTGGGAACTCACGGGGGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGATGA
  B.breve CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGCCCCGTGTTGCCAGCGGATTATGCCGGGAACTCACGGGGGACCGCCGGGGTTAACTCGGAGGAAGGTGGGGATGA
 B.longum CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGCCCCGTGTTGCCAGCGGATTATGCCGGGAACTCACGGGGGACCGCCGGGGTTAACTCGGAGGAAGGTGGGGATGA
     lm26
      lm3
Consensus ..................................................................................................................................

          1171  1180      1190      1200      1210      1220      1230      1240      1250      1260      1270      1280      1290      1300
          |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
B.bifidum CGTCAGATCATCATGCCCCTTACGTCCAGGGCTTCACGCATGCTACAATGGCCGGTACAGCGGGATGCGACATGGCGACATGGAGCGGATCCCTGAAAACCGGTCTCAGTTCGGATCGGAGCCTGCAACC
B.dentium CGTCAGATCATCATGCCCCTTACGTCCAGGGCTTCACGCATGCTACAATGGCCGGTACAGCGGGATGCGACATGGCGACATGGAGCGGATCCCTGAAAACCGGTCTCAGTTCGGATTGGAGTCTGCAACC
  B.breve CGTCAGATCATCATGCCCCTTACGTCCAGGGCTTCACGCATGCTACAATGGCCGGTACAACGGGATGCGACAGTGCGAGCTGGAGCGGATCCCTGAAAACCGGTCTCAGTTCGGATCGCAGTCTGCAACT
 B.longum CGTCAGATCATCATGCCCCTTACGTCCAGGGCTTCACGCATGCTACAATGGCCGGTACAACGGGATGCGACGCGGCGACGCGGAGCGGATCCCTGAAAACCGGTCTCAGTTCGGATCGCAGTCTGCAACT
     lm26
      lm3
Consensus ..................................................................................................................................

          1301  1310      1320      1330      1340      1350      1360      1370      1380      1390      1400      1410      1420      1430
          |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
B.bifidum CGGCTCCGTGAAGGCGGAGTCGCTAGTAATCGCGGATCAGCAACGCCGCGGTGAATGCGTTCCCGGGCCTTGTACACACCGCCCGTCAAGTCATGAAAGTGGGCAGCACCCGAAGCCGGTGGCCTAACCC
B.dentium CGACTCCATGAAGGCGGAGTCGCTAGTAATCGCGGATCAGCAACGCCGCGGTGAATGCGTTCCCGGGCCTTGTACACACCGCCCGTCAAGTCATGAAAGTGGGCAGCACCCGAAGCCGGTGGCCTAACCC
  B.breve CGACTGCGTGAAGGCGGAGTCGCTAGTAATCGCGAATCAGCAACGTCGCGGTGAATGCGTTCCCGGGCCTTGTACACACCGCCCGTCAAGTCATGAAAGTGGGCAGCACCCGAAGCCGGTGGCCTAACCC
 B.longum CGACTGCGTGAAGGCGGAGTCGCTAGTAATCGCGAATCAGCAACGTCGCGGTGAATGCGTTCCCGGGCCTTGTACACACCGCCCGTCAAGTCATGAAAGTGGGCAGCACCCGAAGCCGGTGGCCTAACCC
     lm26
      lm3                                                                                            CATGAAAGTGGG-AGCACCCG
Consensus ...........................................................................................catgaaagtggg.agcacccg..................

          1431  1440      1450      1460      1470      1480      1490      1500      1510      1520  1526
          |--------+---------+---------+---------+---------+---------+---------+---------+---------+-----|
B.bifidum CT-TGTGGGATGGAGCCGTCTAAGGTGAGGCTCGTGATTGGGACTAAGTCGTAACAAGGTAGCCGTACCGGAAGGTGCGGCTGGATCACCTCCTTA
B.dentium TTCTGTGGGATGGAGCCGTCTAAGGTGAGGCTCGTGATTGGGACTAAGTCGTAACAAGGTAGCCGTACCGGAAGGTGCGGCTGGATCACCTCCT
  B.breve CT-TGCGGGAGGGAGCCGTCTAAGGTGAGGCTCGTGATTGGGACTAAGTCGTAACAAGGTAGCCGTACCGGAAGGTGCGGCTGGATCACCTCCTT
 B.longum CT-TGTGGGATGGAGCCGTCTAAGGTGAGGCTCGTGATTGGGACTAAGTCGTAACAAGGTAGCCGTACCGGAAGGTGCGGCTGGATCACCTCCTT
     lm26
      lm3
Consensus .. ............................................................................................
```

Figure 9. The alignment of universal primers lm 26 and lm3 with 16S rRNA gene sequence of *Bifidobacterium* spp.



```
               1       10        20        30        40        50        60        70        80        90       100       110       120       130
               |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
S.thermophilus       AGAGTTTGATCCTGGCTCAGGACGAACGGTGGCGGCGTGCCTAATACATGCAAGTAGAACGCTGAAGAGAGGAGCTTGCTCTTCTTGGATGAGTTGCGAACGGGTGAGTAACGCGTAGGTAAC
S.vestibularis   ATGGGAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTAGAACGCTGAAGAGAGGAGCTTGCTCTTCTTGGATGAGTTGCGAACGGGTGAGTAACGCGTAGGTAAC
  S.salivarius TTTAATGAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTAGAACGCTGAAGAGAGGAGCTTGCTCTTCTTGGATGAGTTGCGAACGGGTGAGTAACGCGTAGGTAAC
       forward       AGAGTTTGATCCTGGCTCAG
          U926
     Consensus .......agagtttgatcctggctcag.......................................................................................................

               131    140       150       160       170       180       190       200       210       220       230       240       250       260
               |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
S.thermophilus CTGCCTNGTAGCGGGGGATAACTATTGGAAACGATAGCTAATACCGCATAACAATGGATGACACATGTCATTTATTTGAAAGGGGCAATTGCTCCACTACAAGATGGACCTGCGTTGTATTAGCTAGTAG
S.vestibularis CTGCCTTGTAGCGGGGGATAACTATTGGAAACGATAGCTAATACCGCATAACAATAGGTGACACATGTCATTTATTTGAAAGGGGCAATTGCTCCACTACAAGATGGACCTGCGTTGTATTAGCTAGTAG
  S.salivarius CTGCCTTGTAGCGGGGGATAACTATTGGAAACGATAGCTAATACCGCATAACAATGGATGACCCATGTCATTTATTTGAAAGGGGCAAATGCTCCACTACAAGATGGACCTGCGTTGTATTAGCTAGTAG
       forward
          U926
     Consensus ..................................................................................................................................

               261    270       280       290       300       310       320       330       340       350       360       370       380       390
               |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
S.thermophilus GTGAGGTAATGGCTTACCTAGGCGACGATACATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGGGGCAAC
S.vestibularis GTGAGGTAACGGCTCACCTAGGCGACGATACATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGGGGCAAC
  S.salivarius GTGAGGTAACGGCTCACCTAGGCGACGATACATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGGGGCAAC
       forward
          U926
     Consensus ..................................................................................................................................

               391    400       410       420       430       440       450       460       470       480       490       500       510       520
               |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
S.thermophilus CCTGACCGAGCAACGCCGCGTGACTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTAAGTCAAGAACGGGTGTGAGAGTGGAAAGTTCACACAGTGACGGTAGCTTACCAGAAAGGGACGGCTAACTA
S.vestibularis CCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTAAGTCAAGAACGAGTGTGAGAGTGGAAAGTTCACACTGTGACGGTAGCTTACCAGAA-GGGACGGCTAACTA
  S.salivarius CCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTAAGTCAAGAACGAGTGTGAGAGTGGAAAGTTCACACTGTGACGGTAGCTTACCAGAAAGGGACGGCTAACTA
       forward
          U926
     Consensus ..................................................................................................................................

               521    530       540       550       560       570       580       590       600       610       620       630       640       650
               |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
S.thermophilus CGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTGATAAGTCTGAAGTTAAAGGCTGTGGCTCAACCATAGTTCGCTTTGGAAACT
S.vestibularis CGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTGATAAGTCTGAAGTTAAAGGCTGTGGCTCAACCATAGTTCGCTTTGGAAACT
  S.salivarius CGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTGATAAGTCTGAAGTTAAAGGCTGTGGCTCAACCATAGTTCGCTTTGGAAACT
       forward
          U926
     Consensus ..................................................................................................................................

               651    660       670       680       690       700       710       720       730       740       750       760       770       780
               |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
S.thermophilus GTCAAACTTGAGTGCAGAAGGGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCGTGG
S.vestibularis GTCAAACTTGAGTGCAGAAGGGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCGTGG
  S.salivarius GTCAAACTTGAGTGCAGAAGGGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCGTGG
       forward
          U926
     Consensus ..................................................................................................................................

               781    790       800       810       820       830       840       850       860       870       880       890       900       910
               |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
S.thermophilus GGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTGGATCCTTTCCGGGATTCAGTGCCGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGGAAGG
S.vestibularis GGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTGGATCCTTTCCGGGATTCAGTGCCGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGG
  S.salivarius GGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTGGATCCTTTCCGGGATTCAGTGCCGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGG
       forward
          U926
     Consensus ..................................................................................................................................

               911    920       930       940       950       960       970       980       990      1000      1010      1020      1030      1040
               |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
S.thermophilus TTGAAACTCAAAGGAATTGACGGGG-CCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCACCTCTTGACATCCCGATGCTATTTCTAGAGATAGAAAGTTACTTTGG
S.vestibularis TTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCCGATGCTATTTCTAGAGATAGAAAGTTACTTCGG
  S.salivarius TTGAAACTCAAAGGAATTGACGGGGGCCNGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCCGATGCTATTTCTAGAGATAGAAAGTTACTTCGG
       forward
          U926    AAACTCAAAGGAATTGACGG
     Consensus ...aaactcaaaggaattgacgg...........................................................................................................
               1041  1050      1060      1070      1080      1090      1100      1110      1120      1130      1140      1150      1160      1170
               |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
S.thermophilus TACATCGGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTATTGTTAGTTGCCATCATTCAGTTGGGCACTCTAGCGAGACTGCCGG
S.vestibularis TACATCGGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTATTGTTAGTTGCCATCATTCAGTTGGGCACTCTAGCGAGACTGCCGG
  S.salivarius TACATCGGTGACAGGTGGNGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTATTGTTAGTTGCCATCATTCAGTTGGGCACTCTAGCGAGACTGCCGG
       forward
          U926
     Consensus ..................................................................................................................................

               1171  1180      1190      1200      1210      1220      1230      1240      1250      1260      1270      1280      1290      1300
               |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
S.thermophilus TAATAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGTTGGTACAACGAGTTGCGAGTCGGTGACGGCGAGCTAATCTCTTAAAGCCAA
S.vestibularis TAATAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGTTGGTACAACGAGTTGCGAGTCGGTGACGGCAAGCTAATCTCTTAAAGCCAA
  S.salivarius TAATAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGTTGGTACAACGAGTTGCGAGTCGGTGACGGCAAGCTAATCTCTTAAAGCCAA
       forward
          U926
     Consensus ..................................................................................................................................

               1301  1310      1320      1330      1340      1350      1360      1370      1380      1390      1400      1410      1420      1430
               |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
S.thermophilus TCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGT
S.vestibularis TCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGT
  S.salivarius TCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGT
       forward
          U926
     Consensus ..................................................................................................................................

               1431  1440      1450      1460      1470      1480      1490      1500      1510      1520      1530      1540       1551
               |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+|
S.thermophilus AACACCCGAAGTCGGTGAGGTAACCTTTTGGAGCCAGCCGCCTAAGGTGGGACAGATGATTGGGGTGAAGTCGTAACAAGGTAGCCGATCCGGAAGGTGCGGCTGGATCACCTCCTTT
S.vestibularis AACACCCGAAGTCGGTGAGGTAACCTTTTGGAGCCAGCCGCCTAAGGTGGGATAGATGATTGGGGTGAAGTCGTAACAAGGTAGCCGTATCGGAAGGTGCGGCTGGATCAC
  S.salivarius AACACCCGAAGTCGGTGAGGTAACCTTTTGGAGCCAGCCGCCTAAGGTGGGATAGATGA--GGGG-------GTAACAAGGTA------TCGGAAG----------ATCACCTCCTTTCTA
       forward
          U926
     Consensus .........................................................................................................................
```

Figure 10. The alignment of universal primers F and U926 with 16S rRNA gene sequence of *Streptococcus* spp.

Table 6. Genotypic identification of *Lactobacillus* spp. based on 16S rRNA gene sequencing

No.	Subject No. (day of lactation)	Bacterial isolates	Match organism (Data bank)	% Identity	Query length (bp)
1	20 (60d.)	St8	*Lactobacillus gasseri* (NCBI) *Lactobacillus gasseri* (RDP)	99.0 98.9	906
2	25 (60d.)	Lac39	*Lactobacillus salivarius* (NCBI) *Lactobacillus salivarius* (RDP)	98.0 98.5	1513
3	28 (60d.)	Lac31	*Lactobacillus fermentum* (NCBI) *Lactobacillus fermentum* (RDP)	98.0 97.9	928
4	29 (60d.)	Lac40	*Lactobacillus salivarius* (NCBI) *Lactobacillus salivarius* (RDP)	99.0 99.4	1477
5	30 (60d.)	Lac41	*Lactobacillus salivarius* (NCBI) *Lactobacillus salivarius* (RDP)	100.0 99.6	656
6	35 (15d.)	Lac42	*Lactobacillus salivarius* (NCBI) *Lactobacillus salivarius* (RDP)	100.0 99.8	917
7	40 (15d.)	Lac43	*Lactobacillus rhamnosus* (NCBI) *Lactobacillus rhamnosus* (RDP)	98.0 98.4	1507
8	41 (15d.)	Lac44	*Lactobacillus casei* (NCBI) *Lactobacillus casei* (RDP)	99.0 98.9	786
9	44 (15d.)	Lac45	*Lactobacillus salivarius* (NCBI) *Lactobacillus salivarius* (RDP)	94.0 94.6	624

Table 6. Genotypic identification of *Lactobacillus* spp. based on 16S rRNA gene sequencing (Continued)

No.	**Subject No.** (day of lactation)	**Bacterial isolates**	**Match organism (Data bank)**	**% Identity**	**Query length (bp)**
10	45 (60d.)	NL1	*Lactobacillus salivarius* (NCBI)	99.0	1101
			Lactobacillus salivarius (RDP)	98.9	
		NL2	*Lactobacillus salivarius* (NCBI)	100.0	1157
			Lactobacillus salivarius (RDP)	99.6	
		NL3	*Lactobacillus salivarius* (NCBI)	99.0	1497
			Lactobacillus salivarius (RDP)	99.5	
11	50 (60d.)	NL5	*Lactobacillus salivarius* (NCBI)	100.0	653
			Lactobacillus salivarius (RDP)	99.8	
12	54 (30d.)	NL6	*Lactobacillus salivarius* (NCBI)	99.0	614
			Lactobacillus salivarius (RDP)	98.9	
		NL7	*Lactobacillus salivarius* (NCBI)	82.0	796
			Lactobacillus salivarius (RDP)	82.4	
13	55 (60d.)	NL8	*Lactobacillus gasseri* (NCBI)	100.0	1493
			Lactobacillus gasseri (RDP)	99.8	
14	56 (60d.)	NL9	*Lactobacillus salivarius* (NCBI)	97.0	1311
			Lactobacillus salivarius (RDP)	97.4	
15	59 (28d.)	NL10	*Lactobacillus gasseri* (NCBI)	99.0	901
			Lactobacillus gasseri (RDP)	99.5	

Table 6. Genotypic identification of *Lactobacillus* spp. based on 16S rRNA gene sequencing (Continued)

No.	Subject No. (day of lactation)	Bacterial isolates	Match organism (Data bank)	% Identity	Query length (bp)
16	61 (30d.)	NL12	*Lactobacillus salivarius* (NCBI) *Lactobacillus salivarius* (RDP)	98.0 97.8	1028
17	62 (60d.)	NL16	*Lactobacillus fermentum* (NCBI) *Lactobacillus fermentum* (RDP)	99.0 98.9	903
18	63 (60d.)	NL18	*Lactobacillus mucosae* (NCBI) *Lactobacillus mucosae* (RDP)	98.0 98.4	1502
19	65 (30d.)	NL19	*Lactobacillus salivarius* (NCBI) *Lactobacillus salivarius* (RDP)	99.0 99.5	1389
20	67 (60d.)	NL20	*Lactobacillus mucosae* (NCBI) *Lactobacillus mucosae* (RDP)	98.0 97.8	962
21	70 (60d.)	NL25	*Lactobacillus mucosae* (NCBI) *Lactobacillus mucosae* (RDP)	97.0 96.9	1523
22	71 (60d.)	NL26	*Lactobacillus salivarius* (NCBI) *Lactobacillus salivarius* (RDP)	98.0 97.8	661
23	72 (60d.)	NL45	*Lactobacillus salivarius* (NCBI) *Lactobacillus salivarius* (RDP)	94.0 93.9	601
24	75 (60d.)	NL46	*Lactobacillus mucosae* (NCBI) *Lactobacillus mucosae* (RDP)	100.0 100.0	1077

Table 6. Genotypic identification of *Lactobacillus* spp. based on 16S rRNA gene sequencing (Continued)

No.	**Subject No.** (day of lactation)	**Bacterial isolates**	**Match organism (Data bank)**	**% Identity**	**Query length (bp)**
25	76 (60d.)	NL48	*Lactobacillus gasseri* (NCBI)	99.0	1296
			Lactobacillus gasseri (RDP)	99.4	
26	78 (60d.)	NL49	*Lactobacillus oris* (NCBI)	95.0	621
			Lactobacillus oris (RDP)	94.8	
27	81 (60d.)	NL50	*Lactobacillus fermentum* (NCBI)	97.0	747
			Lactobacillus fermentum (RDP)	97.4	
28	84 (60d.)	NL52	*Lactobacillus mucosae* (NCBI)	99.0	1509
			Lactobacillus mucosae (RDP)	98.6	
29	86 (60d.)	NL53	*Lactobacillus fermentum* (NCBI)	100.0	749
			Lactobacillus fermentum (RDP)	99.8	
30	87 (23d.)	NL54	*Lactobacillus gasseri* (NCBI)	97.0	809
			Lactobacillus gasseri (RDP)	97.4	
31	90 (60d.)	NL55	*Lactobacillus fermentum* (NCBI)	97.0	780
			Lactobacillus fermentum (RDP)	96.9	
32	92 (60d.)	NL56	*Lactobacillus rhamnosus* (NCBI)	99.0	948
			Lactobacillus rhamnosus (RDP)	98.9	

Table 6. Genotypic identification of *Lactobacillus* spp. based on 16S rRNA gene sequencing (Continued)

No.	**Subject No.** (day of lactation)	**Bacterial isolates**	**Match organism (Data bank)**	**% Identity**	**Query length (bp)**
33	95 (30d.)	NL57	*Lactobacillus casei* (NCBI)	85.0	791
			Lactobacillus casei (RDP)	85.4	
34	97 (60d.)	NL58	*Lactobacillus rhamnosus* (NCBI)	97.0	759
			Lactobacillus rhamnosus (RDP)	97.5	
35	98 (60d.)	NL60	*Lactobacillus casei* (NCBI)	98.0	658
			Lactobacillus casei (RDP)	98.4	
36	100 (60d.)	NL61	*Lactobacillus plantarum* (NCBI)	99.0	942
			Lactobacillus plantarum (RDP)	98.6	
37	102 (60d.)	NL62	*Lactobacillus gasseri* (NCBI)	98.0	986
			Lactobacillus gasseri (RDP)	97.9	

Table 7. Genotypic identification of *Bifidobacterium* spp. based on 16S rRNA gene sequencing

No.	**Subject No.** (day of lactation)	**Bacterial isolates**	**Match organism (Data bank)**	**% Identity**	**Query length (bp)**
1	23 (60d.)	Bif29	*Bifidobacterium breve* (NCBI)	100.0	874
			Bifidobacterium breve (RDP)	99.8	
2	26 (22d.)	NB1	*Bifidobacterium breve* (NCBI)	99.0	1355
			Bifidobacterium breve (RDP)	99.8	
3	31 (15d.)	NB2	*Bifidobacterium psedocatenulatum* (NCBI)	99.0	1360
			Bifidobacterium psedocatenulatum (RDP)	99.5	
4	40 (15d.)	NB3	*Bifidobacterium dentium* (NCBI)	99.0	1355
			Bifidobacterium dentium (RDP)	99.8	
5	41 (15d.)	NB4	*Bifidobacterium dentium* (NCBI)	99.0	1358
			Bifidobacterium dentium (RDP)	99.5	
6	45 (60d.)	NB5	*Bifidobacterium bifidum*(NCBI)	99.0	1056
			Bifidobacterium bifidum (RDP)	99.8	
		NB6	*Bifidobacterium dentium* (NCBI)	99.0	1356
			Bifidobacterium dentium (RDP)	99.5	
7	47 (60d.)	NB8	*Bifidobacterium longum* (NCBI)	99.0	1340
			Bifidobacterium longum (RDP)	99.8	

Table 7. Genotypic identification of *Bifidobacterium* spp. based on 16S rRNA gene sequencing (Continued)

No.	Subject No. (day of lactation)	Bacterial isolates	Match organism (Data bank)	% Identity	Query length (bp)
8	48 (60d.)	NB9	*Bifidobacterium psedocatenulatum* (NCBI) *Bifidobacterium psedocatenulatum* (RDP)	98.0 98.4	1356
9	53 (30d.)	NB10	*Bifidobacterium psedocatenulatum* (NCBI) *Bifidobacterium psedocatenulatum* (RDP)	98.0 98.6	1356
10	54 (30d.)	NB11	*Bifidobacterium dentium* (NCBI) *Bifidobacterium dentium* (RDP)	100.0 99.8	993
11	59 (28d.)	NB12	*Bifidobacterium bifidum* (NCBI) *Bifidobacterium bifidum* (RDP)	98.0 98.4	973
12	61 (30d.)	NB13	*Bifidobacterium bifidum* (NCBI) *Bifidobacterium bifidum* (RDP)	100.0 99.5	938
13	64 (30d.)	NB14	*Bifidobacterium dentium* (NCBI) *Bifidobacterium dentium* (RDP)	100.0 99.8	1475
14	67 (60d.)	NB15	*Bifidobacterium bifidum* (NCBI) *Bifidobacterium bifidum* (RDP)	99.0 98.9	1365

Table 7. Genotypic identification of *Bifidobacterium* spp. based on 16S rRNA gene sequencing (Continued)

No.	**Subject No.** (day of lactation)	**Bacterial isolates**	**Match organism (Data bank)**	**% Identity**	**Query length (pb)**
15	70 (60d.)	NB16	*Bifidobacterium bifidum* (NCBI)	99.0	1350
			Bifidobacterium bifidum (RDP)	98.8	
16	71 (60d.)	NB17	*Bifidobacterium longum* (NCBI)	98.0	1347
			Bifidobacterium longum (RDP)	98.6	
17	72 (60d.)	NB18	*Bifidobacterium longum* (NCBI)	99.0	696
			Bifidobacterium longum (RDP)	99.5	
18	75 (60d.)	NB19	*Bifidobacterium longum* (NCBI)	99.0	985
			Bifidobacterium longum (RDP)	98.8	
19	76 (60d.)	NB20	*Bifidobacterium longum* (NCBI)	99.0	1350
			Bifidobacterium longum (RDP)	98.9	
20	82 (60d.)	NB25	*Bifidobacterium breve* (NCBI)	99.0	623
			Bifidobacterium breve (RDP)	98.8	
21	83 (60d.)	NB28	*Bifidobacterium longum* (NCBI)	99.0	1350
			Bifidobacterium longum (RDP)	99.5	
22	85 (60d.)	NB31	*Bifidobacterium breve* (NCBI)	98.0	701
			Bifidobacterium breve (RDP)	98.4	
		NB37	*Bifidobacterium dentium* (NCBI)	98.0	1355
			Bifidobacterium dentium (RDP)	98.6	

Table 7. Genotypic identification of *Bifidobacterium* spp. based on 16S rRNA gene sequencing (Continued)

No.	**Subject No.** (day of lactation)	**Bacterial isolates**	**Match organism (Data bank)**	**% Identity**	**Query length (bp)**
23	86 (60d.)	NB38	*Bifidobacterium breve* (NCBI) *Bifidobacterium breve* (RDP)	100.0 99.8	1368
24	87 (60d.)	NB39	*Bifidobacterium bifidum* (NCBI) *Bifidobacterium bifidum* (RDP)	98.0 98.8	1460
25	90 (60d.)	NB40	*Bifidobacterium dentium* (NCBI) *Bifidobacterium dentium* (RDP)	100.0 99.5	1365
26	91 (60d.)	NB42	*Bifidobacterium bifidum* (NCBI) *Bifidobacterium bifidum* (RDP)	100.0 99.8	1222
27	92 (60d.)	NB45	*Bifidobacterium psedocatenulatum* (NCBI) *Bifidobacterium psedocatenulatum* (RDP)	99.0 98.9	1351
28	95 (60d.)	NB46	*Bifidobacterium breve* (NCBI) *Bifidobacterium breve* (RDP)	98.0 98.5	1363
29	97 (60d.)	NB47	*Bifidobacterium longum* (NCBI) *Bifidobacterium longum* (RDP)	99.0 99.4	1540

Table 7. Genotypic identification of *Bifidobacterium* spp. based on 16S rRNA gene sequencing (Continued)

No.	Subject No. (day of lactation)	Bacterial isolates	Match organism (Data bank)	% Identity	Query length (bp)
30	98 (60d.)	NB48	*Bifidobacterium psedocatenulatum* (NCBI)	99.0	1369
			Bifidobacterium psedocatenulatum (RDP)	98.8	
31	102 (60d.)	NB49	*Bifidobacterium breve* (NCBI)	97.0	1355
			Bifidobacterium breve (RDP)	97.5	

Table 8. Genotypic identification of *Streptococcus* spp. based on 16S rRNA gene sequencing

No.	**Subject No.** (day of lactation)	**Bacterial isolates**	**Match organism (Data bank)**	**% Identity**	**Query length (bp)**
1	2 (15d.)	St1	*Streptococcus salivarius* (NCBI)	98.0	875
			Streptococcus salivarius (RDP)	97.9	
2	8 (15d.)	St2	*Streptococcus salivarius* (NCBI)	98.0	765
			Streptococcus salivarius (RDP)	98.4	
3	10 (15d.)	St4	*Streptococcus* sp. (NCBI)	99.0	914
			Streptococcus sp. (RDP)	98.9	
4	17 (60d.)	St5	*Streptococcus salivarius* (NCBI)	98.0	886
			Streptococcus salivarius (RDP)	98.6	
5	18 (60d.)	St6	*Streptococcus salivarius* (NCBI)	98.0	888
			Streptococcus salivarius (RDP)	97.9	
		St7	*Streptococcus mitis* (NCBI)	98.0	893
			Streptococcus mitis (RDP)	98.3	
6	21 (60d.)	Lac22	*Streptococcus lactarius* (NCBI)	100.0	669
			Streptococcus lactarius (RDP)	99.5	
		St9	*Streptococcus parasanguis* (NCBI)	98.0	887
			Streptococcus parasanguis (RDP)	97.6	

Table 8. Genotypic identification of *Streptococcus* spp. based on 16S rRNA gene sequencing (Continued)

No.	Subject No. (day of lactation)	Bacterial isolates	Match organism (Data bank)	% Identity	Query length (pb)
7	22 (60d.)	St10	*Streptococcus lactarius* (NCBI)	99.0	886
			Streptococcus lactarius (RDP)	99.4	
		St11	*Streptococcus lactarius* (NCBI)	99.0	883
			Streptococcus lactarius (RDP)	98.6	
		St12	*Streptococcus salivarius* (NCBI)	98.0	884
			Streptococcus salivarius (RDP)	97.9	
		St13	*Streptococcus salivarius* (NCBI)	98.0	887
			Streptococcus salivarius (RDP)	97.6	
		St14	*Streptococcus salivarius* (NCBI)	98.0	888
			Streptococcus salivarius (RDP)	98.3	
8	23 (60d.)	St15	*Streptococcus salivarius* (NCBI)	98.0	886
			Streptococcus salivarius (RDP)	98.5	
		St16	*Streptococcus mitis* (NCBI)	99.0	884
			Streptococcus mitis (RDP)	98.9	
9	50 (60d.)	NL4	*Streptococcus* sp. (NCBI)	99.0	935
			Streptococcus sp. (RDP)	99.5	
10	54 (30d.)	NL9	*Streptococcus salivarius* (NCBI)	97.0	931
			Streptococcus salivarius (RDP)	97.4	

Table 8. Genotypic identification of *Streptococcus* spp. based on 16S rRNA gene sequencing (Continued)

No.	**Subject No.** (day of lactation)	**Bacterial isolates**	**Match organism (Data bank)**	**% Identity**	**Query length (bp)**
11	61 (30d.)	St19	*Streptococcus salivarius* (NCBI)	95.0	864
			Streptococcus salivarius (RDP)	94.6	
12	67 (60days)	St20	*Streptococcus salivarius* (NCBI)	94.0	865
			Streptococcus salivarius (RDP)	94.5	
13	70 (60d.)	St21	*Streptococcus salivarius* (NCBI)	95.0	838
			Streptococcus salivarius (RDP)	94.9	
		St22	*Streptococcus parasanguis* (NCBI)	97.0	824
			Streptococcus parasanguis (RDP)	97.4	
14	81 (60d.)	St26	*Streptococcus lactarius* (NCBI)	99.0	849
			Streptococcus lactarius (RDP)	99.5	
15	87 (23d.)	St27	*Streptococcus* sp. (NCBI)	99.0	855
			Streptococcus sp. (RDP)	98.9	
16	95 (30d.)	St32	*Streptococcus* sp. (NCBI)	99.0	846
			Streptococcus sp. (RDP)	99.4	
		St33	*Streptococcus mitis* (NCBI)	97.0	641
			Streptococcus mitis (RDP)	96.9	
17	100 (60d.)	St34	*Streptococcus salivarius* (NCBI)	92.0	262
			Streptococcus salivarius (RDP)	91.8	

Table 9. Bacterial isolates cultivated from breast milk samples and genotypically identified

Culture	**No. of isolates**	**No. of samples (Total=102)**
MRS culture:		
Suspected *Lactobacillus*	74	71
Positive by *Lactobacillus*-specific PCR	53	49
Identified as *Lactobacillus* spp. based on 16S rRNA sequencing	40	37
MC culture:		
Suspected *Bifidobacterium*	62	58
Positive by *Bifidobacterium* -specific PCR	45	43
Identified as *Bifidobacterium* spp. based on 16S rRNA sequencing	33	31
M17 culture:		
Suspected *Streptococcus*	40	34
Positive by *Streptococcus*-specific PCR	26	23
Identified as *Streptococcus* spp. based on 16S rRNA sequencing	26	17

Table 10. *Lactobacillus, Bifidobacterium* and *Streptococcus* species in breast milk identified by 16S rRNA gene sequencing

Bacterial species	**Number of isolates**	**Occurrence in milk samples**
Lactobacillus		
L. gasseri	6	6
L. salivarius	16	13
L. fermentum	5	5
L. mucosae	5	5
L. rhamnosus	3	3
L. casei	3	3
L. plantarum	1	1
L. oris	1	1
Total	**40**	**37 (36.27%)**
Bifidobacterium		
B. longum	8	8
B. breve	7	6
B. psedocatenulatum	5	5
B. dentium	8	7
B. bifidum	5	5
Total	**33**	**31 (30.39%)**
Streptococcus		
S. salivarius	13	10
S. lactarius	4	3
Streptococcus sp.	4	4
S. mitis	3	3
S. parasangius	2	2
Total	**26**	**17 (16.67%)**

Table 11. Other bacterial species found in breast milk

Groups of bacteria	No. of isolates
Staphylococcus aureus	5
Staphylococcus epidermidis	4
Actinomyces radicidentis	1
uncultured bacteria	15
Total	**25**

3. PCR analyses of lactobacilli, bifidobacteria and streptococci from breast milk samples.

Since culture may not be able to recover all lactobacilli, bifidobacteria and streptococci, PCR was used to detect the presence of these bacteria in breast milk samples. DNA targets were extracted from samples using QIAamp DNA stool minikit (Qiagen, Hilden, Germany). DNA target were amplified by genus-specific primers L159F and L677R for *Lactobacillus* spp., Bif164F and Bif601R for *Bifidobacterium* spp. and Tuf-Strep-1 and Tuf-Strep-R for *Streptococcus* spp. The amplified product of *Lactobacillus*, *Bifidobacterium* and *Streptococcus* was 546bp, 443bp and 560bp, respectively (Figure 11). Lactobacilli, bifidobacteria and streptococci DNA was detected by PCR in 94 (92.16%), 60 (58.82%) and 56 (54.90%) out of 102 breast milk samples, respectively (Figure 12).

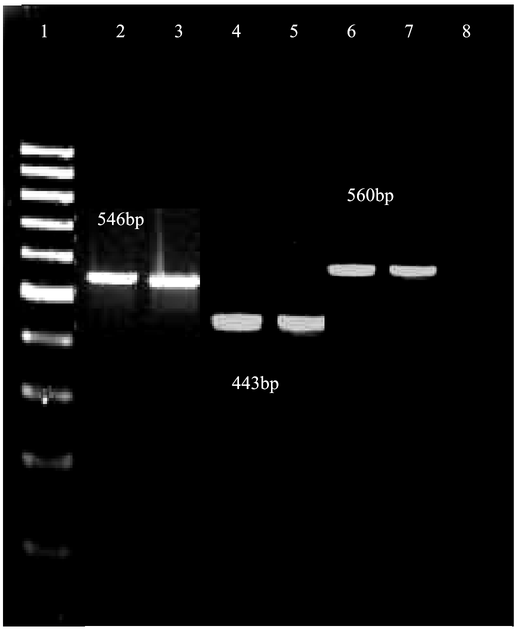


Figure 11. Genus-specific amplification by Polymerase Chain Reaction (PCR). Lane 1, 100 bp DNA ledder: lane 2, suspected *Lactobacillus* sample: lane 3, *L. salivarius* control: lane 4, suspected *Bifidobacterium* sample: lane 5, *B. bifidum* control: lane 6, suspected *Streptococcus* sample: lane 7, *S. salivarius* control: lane 8, negative control.

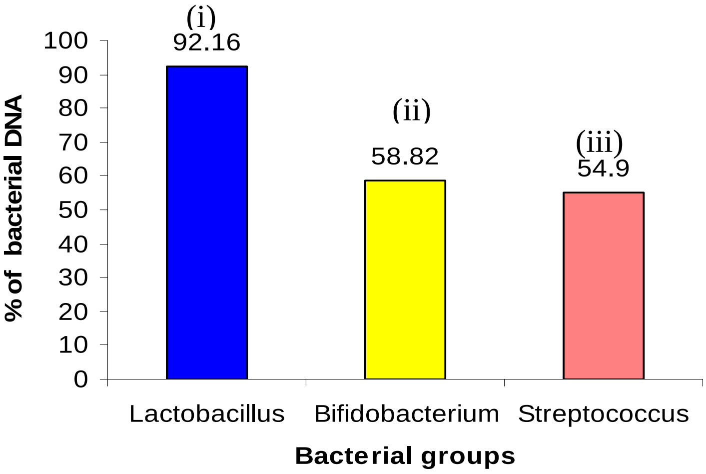


Figure 12. Percentage of bacterial DNA detected by PCR using genus-specific primers. (ii) *Lactobacillus* , (ii) *Bifidobacterium* and (iii) *Streptococcus*

4. Antagonistic activity of *Lactobacillus*, *Bifidobacterium* and *Streptococcus* isolates against bacterial pathogens

The inhibitory activity of 40 *Lactobacillus* isolates was demonstrated in Table 12. All 40 *Lactobacillus* isolates had no inhibitory effect against *E. coli* and *H. pylori* but these isolates had weak inhibition against ETEC, EIEC, EPEC, EHEC and *S.* Typhimurium. Out of 40 isolates, 30 (75.00%) were able to inhibit the growth of *V. cholerae* and *S. flexneri*. The strong inhibitory activity of *Lactobacillus* against *V. cholerae* and *S. flexneri* was found in 9 isolates (Lac43, Lac44, Lac45, NL1, NL6, NL7, NL8, NL18 and NL50) and 6 isolates (Lac45, NL3, NL5, NL6, NL10 and NL50), respectively. The clear zones demonstrated antagonistic activity of lactobacilli against *V. cholerae* was shown in Figure 13. Furthermore, six *Lactobacillus* isolates had weak inhibition against Methicillin-resistant *Staphylococcus aureus* (MRSA) as shown in Figure 14.

The inhibitory activity of 33 *Bifidobacterium* isolates was demonstrated in Table 13. All *Bifidobacterium* isolates had no inhibition against *E. coli* and MRSA but they had partial inhibition against ETEC, EIEC, EPEC, EHEC and *S.* Typhimurium as microcolony. Out of 33 isolates, 30 (90.91%) and 28 (84.85%) were able to inhibit the growth of *V. cholerae* and *S. flexneri*, respectively. The strong inhibitory activity of *Bifidobacterium* against *V. cholerae* and *S. flexneri* found as 10 isolates (Bif29, NB4, NB11, NB13, NB14, NB15, NB16, NB17, NB31 and NB40) and 1 isolate (NB28), respectively. The clear zones demonstrated antagonistic activity of bifidobacteria against *V. cholerae* were shown in Figure 15. In addition, 23 (69.70%) out of 33 isolates inhibited the growth of *H. pylori*. Five of these 23 *Bifidobacterium* isolates (NB6, NB8, NB14, NB28 and NB31) had strong inhibitory activities against *H. pylori*.

The inhibitory activities of *Streptococcus* were tested in 21 isolates whereas those of 5 isolates were neglected. These 5 isolates belonged to *Streptococcus mitis* and *Streptococcus parasanguis* which were considered as human pathogens. The inhibitory activities of *Streptococcus* were demonstrated in Table 14. All 21 *Streptococcus* isolates had no inhibition against all bacterial pathogens, except four isolates had partial inhibition against *S. flexneri* as microcolony. Moreover, 8

(36.36%) and 5 (22.73%) out of 21 isolates had weak inhibition against *V. cholerae* and MRSA, respectively. The summary antagonistic activity of *Lactobacillus*, *Bifidobacterium* and *Streptococcus* were shown in Tables 15-17.

Table 12. *Lactobacillus* antagonistic activity against bacterial pathogens

Isolates	**Pathogens**									
	EHEC DMST 12743	EIEC DMST 20971	EPEC DMST 20972	ETEC DMST 20970	*S.* Typhimurium ATCC 13311	*S. flexneri* DMST 4423	V. *cholerae* non O1 DMST 2873	*E. coli* 25922	*H. pylori* ATCC 43504	MRSA ATCC 43300
St8	-	-	-	-	-	-	-	-	-	-
Lac31	-	-	-	-	-	-	-	-	-	-
Lac39	M	M	M	-	M	weak	weak	-	-	-
Lac40	M	M	M	-	M	weak	weak	-	-	strong
Lac41	weak	weak	weak	-	M	weak	weak	-	-	strong
Lac42	-	-	-	-	-	M	M	-	-	-
Lac43	-	M	M	-	-	weak	strong	-	-	-
Lac44	-	M	M	-	weak	weak	strong	-	-	-
Lac45	weak	weak	weak	M	weak	weak	strong	-	-	-
NL1	weak	weak	M	M	weak	weak	strong	-	-	-
NL2	-	M	M	-	M	weak	weak	-	-	-
NL3	-	M	M	M	M	weak	weak	-	-	-

- : No inhibition zone; M, microcolonies : opaque zone of inhibition <1 mm; weak: a clear inhibition zone of 1-2 mm; strong: a clear inhibition zone of 3-4 mm

Table 12. *Lactobacillus* antagonistic activity against bacterial pathogens (Continued)

Isolates	**Pathogens**									
	EHEC DMST 12743	EIEC DMST 20971	EPEC DMST 20972	ETEC DMST 20970	*S.* Typhimurium ATCC 13311	*S. flexneri* DMST 4423	V. *cholerae* non O1 DMST 2873	*E. coli* 25922	*H. pylori* ATCC 43504	MRSA ATCC 43300
NL5	M	-	M	M	M	strong	weak	-	-	-
NL6	weak	weak	weak	weak	weak	strong	strong	-	-	-
NL7	weak	weak	weak	M	weak	weak	strong	-	-	-
NL8	-	-	-	-	-	weak	strong	-	-	-
NL9	weak	weak	M	-	M	weak	weak	-	-	-
NL10	-	M	-	-	-	strong	weak	-	-	-
NL12	-	M	-	-	-	weak	weak	-	-	-
NL16	-	-	-	-	-	M	weak	-	-	-
NL18	-	-	-	-	-	weak	strong	-	-	weak
NL19	M	-	M	-	-	weak	weak	-	-	-
NL20	-	-	-	-	-	-	-	-	-	-
NL25	-	M	-	M	-	weak	weak	-	-	-

- : No inhibition zone; M, microcolonies : opaque zone of inhibition <1 mm; weak: a clear inhibition zone of 1-2 mm; strong: a clear inhibition zone of 3-4 mm

Table 12. *Lactobacillus* antagonistic activity against bacterial pathogens (Continued)

Isolates	**Pathogens**									
	EHEC DMST 12743	EIEC DMST 20971	EPEC DMST 20972	ETEC DMST 20970	*S.* Typhimurium ATCC 13311	*S. flexneri* DMST 4423	V. *cholerae* non O1 DMST 2873	*E. coli* 25922	*H. pylori* ATCC 43504	MRSA ATCC 43300
NL49	-	-	-	-	-	-	-	-	-	-
NL50	-	-	-	-	-	strong	strong	-	-	weak
NL52	-	-	-	-	-	M	weak	-	-	weak
NL53	-	M	-	-	-	M	weak	-	-	weak
NL54	-	-	M	-	-	-	-	-	-	-
NL55	-	-	-	-	-	-	M	-	-	-
NL56	M	M	M	-	M	M	weak	-	-	-
NL57	M	M	M	-	M	weak	weak	-	-	-
NL58	M	M	M	M	M	weak	weak	-	-	-
NL60	-	M	M	M	-	weak	weak	-	-	-
NL61	M	M	M	M	M	weak	weak	-	-	-
NL62	-	-	-	-	-	-	-	-	-	-

- : No inhibition zone; M, microcolonies : opaque zone of inhibition <1 mm; weak: a clear inhibition zone of 1-2 mm; strong: a clear inhibition zone of 3-4 mm

Table 13. *Bifidobacterium* antagonistic activity against bacterial pathogens

Isolates	**Pathogens**									
	EHEC DMST 12743	EIEC DMST 20971	EPEC DMST 20972	ETEC DMST 20970	*S.* Typhimurium ATCC 13311	*S. flexneri* DMST 4423	V. *cholerae* non O1 DMST 2873	*E. coli* 25922	*H. pylori* ATCC 43504	MRSA ATCC 43300
Bif 29	M	M	M	M	M	weak	strong	-	weak	-
NB1	-	-	-	-	-	-	-	-	-	-
NB2	-	-	-	-	-	weak	weak	-	weak	-
NB3	-	-	-	-	-	weak	weak	-	weak	-
NB4	-	-		-	-	weak	strong	-	weak	-
NB5	M	M	-	-	M	weak	weak	-	weak	-
NB6	-	-	-	-	M	weak	weak	-	strong	-
NB8	-	-	-	-	-	weak	weak	-	strong	-
NB9	-	-	-	-	-	weak	weak	-	weak	-
NB10	-	-	-	-	-	weak	weak	-	-	-
NB11	-	-	-		-	weak	strong	-	weak	-

- : No inhibition zone; M, microcolonies : opaque zone of inhibition <1 mm; weak: a clear inhibition zone of 1-2 mm; strong: a clear inhibition zone of 3-4 mm

Table 13. *Bifidobacterium* antagonistic activity against bacterial pathogens (Continued)

Isolates	Pathogens									
	EHEC DMST 12743	EIEC DMST 20971	EPEC DMST 20972	ETEC DMST 20970	*S.* Typhimurium ATCC 13311	*S. flexneri* DMST 4423	V. *cholerae* non O1 DMST 2873	*E. coli* 25922	*H. pylori* ATCC 43504	MRSA ATCC 43300
NB12	-	M	M	-	-	weak	weak	-	-	-
NB13	-	M	-	M	M	weak	strong	-	weak	-
NB14	-	-	-	-	-	weak	strong	-	strong	-
NB15	-	M	-	M	M	weak	strong	-	weak	-
NB16	-	M	-	-	-	weak	strong	-	weak	-
NB17	-	M	-	-	M	weak	strong	-	weak	-
NB18	M	M		-	M	M	weak		weak	
NB19	M	M	-	M	M	weak	weak	-	-	-
NB20	-	M	-	-	M	-	weak	-	weak	-
NB25	-	-	-	-	-	weak	weak	-	-	-
NB28	-	-	-	-	-	strong	weak	-	strong	-

- : No inhibition zone; M, microcolonies : opaque zone of inhibition <1 mm; weak: a clear inhibition zone of 1-2 mm; strong: a clear inhibition zone of 3-4 mm

Table 13. *Bifidobacterium* antagonistic activity against bacterial pathogens (Continued)

Isolates	Pathogens									
	EHEC DMST 12743	EIEC DMST 20971	EPEC DMST 20972	ETEC DMST 20970	*S.* Typhimurium ATCC 13311	*S. flexneri* DMST 4423	V. *cholerae* non O1 DMST 2873	*E. coli* 25922	*H. pylori* ATCC 43504	MRSA ATCC 43300
NB31	-	M	M	-	M	M	strong	-	strong	-
NB37	-	-	-	-	-	M	weak	-	-	-
NB38	-	-	-	-	-	weak	weak	-	-	-
NB39	M	M	M	M	M	weak	weak	-	weak	-
NB40	-	-	-	-	M	weak	strong	-	weak	-
NB42	-	-	-	-	-	M	weak	-	weak	-
NB45	-	-	-	-	M	M	weak	-	weak	-
NB46	-	-	-	-	-	-	-	-	-	-
NB47	-	-	-	-	-	-	-	-	-	-
NB48	-	-	-	-	-	M	weak	-	weak	-
NB49	-	-	-	-	-	-	M	-	-	-

- : No inhibition zone; M, microcolonies : opaque zone of inhibition <1 mm; weak: a clear inhibition zone of 1-2 mm; strong: a clear inhibition zone of 3-4 mm

Table 14. *Streptococcus* antagonistic activity against bacterial pathogens

Isolates	**Pathogens**									
	EHEC DMST 12743	EIEC DMST 20971	EPEC DMST 20972	ETEC DMST 20970	*S.* Typhimurium ATCC 13311	*S. flexneri* DMST 4423	*V. cholerae* non O1 DMST 2873	*E. coli* 25922	*H. pylori* ATCC 43504	MRSA ATCC 43300
St1	-	-	-	-	-	-	-	-	-	-
St2	-	-	-	-	-	-	-	-	-	-
St4	-	-	-	-	-	-	-	-	-	-
St5	-	-	-	-	-	-	M	-	-	-
St6	-	-	-	-	-	-	weak	-	-	-
Lac22	-	-	-	-	-	-	-	-	-	-
St10	-	-	-	-	-	-	-	-	-	weak
St11	-	-	-	-	-	-	-	-	-	weak
St12	-	-	-	-	-	M	weak	-	-	M
St13	-	-	-	-	-	M	weak	-	-	M
St14	-	-	-	-	-	M	weak	-	-	-

- : No inhibition zone; M, microcolonies : opaque zone of inhibition <1 mm; weak: a clear inhibition zone of 1-2 mm; strong: a clear inhibition zone of 3-4 mm

Table 14. *Streptococcus* antagonistic activity against bacterial pathogens. (Continued)

Isolates	**Pathogens**									
	EHEC DMST 12743	EIEC DMST 20971	EPEC DMST 20972	ETEC DMST 20970	*S.* Typhimurium ATCC 13311	*S. flexneri* DMST 4423	V. *cholerae* non O1 DMST 2873	*E. coli* 25922	*H. pylori* ATCC 43504	MRSA ATCC 43300
St15	-	-	-	-	-	-	weak	-	-	-
NL4	-	-	-	-	-	-	-	-	-	weak
NL9	-	-	-	-	-	-	-	-	-	weak
St19	-	-	-	-	-	-	-	-	-	M
St20	-	-	-	-	-	-	-	-	-	-
St21	-	-	-	-	-	-	-	-	-	M
St26	-	-	-	-	-	-	-			-
St27	-	-	-	-	-	-	weak	-	-	weak
St32	-	-	-	-	-	-	weak	-	-	M
St34	-	-	-	-	-	M	weak	-	-	M

- : No inhibition zone; M, microcolonies : opaque zone of inhibition <1 mm; weak: a clear inhibition zone of 1-2 mm; strong: a clear inhibition zone of 3-4 mm

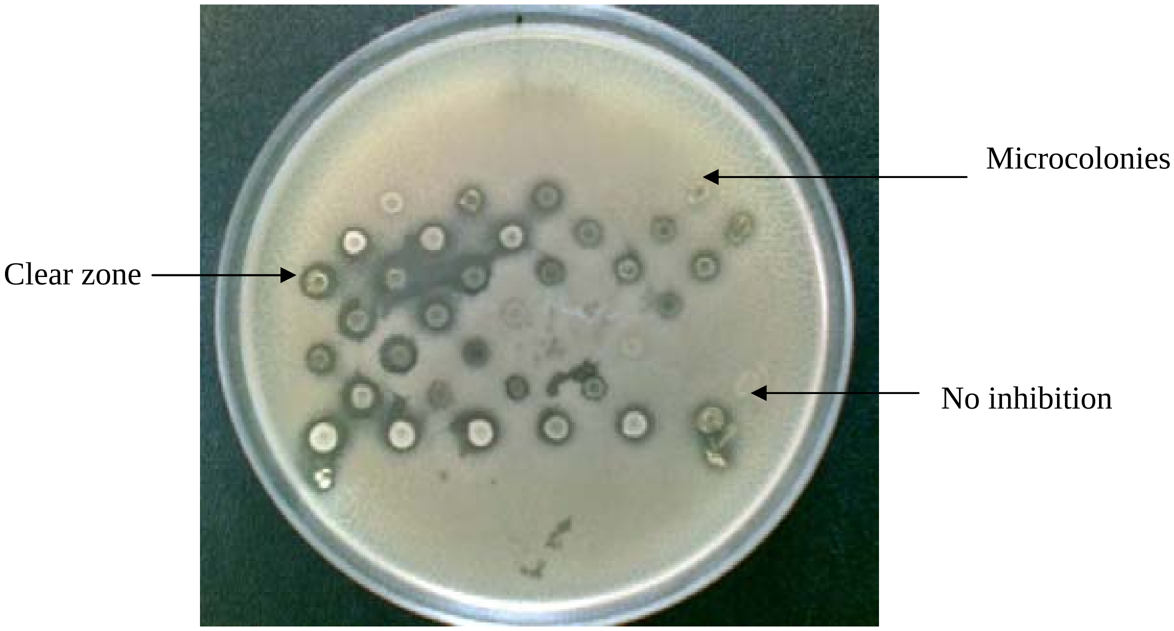


Figure 13. Antagonistic activity of *Lactobacillus* against *Vibrio cholerae* with clear zone, microcolonies and no inhibition zone in 140 mm plate

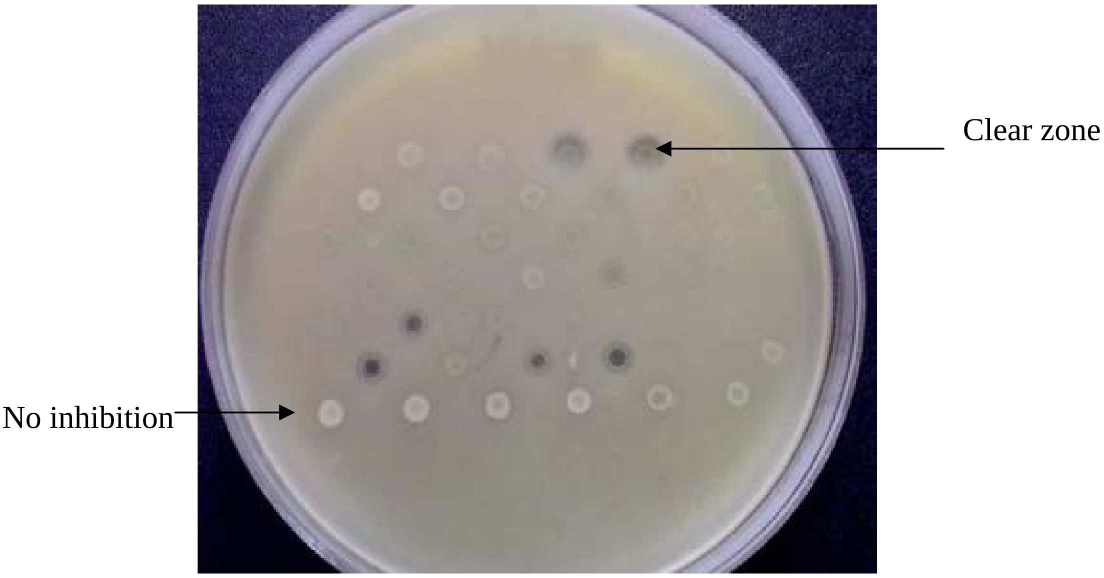


Figure 14. Antagonistic activity of *Lactobacillus* against methicillin-resistant *Staphylococcus aureus* (MRSA) with clear zone and no inhibition zone in 140 mm plate

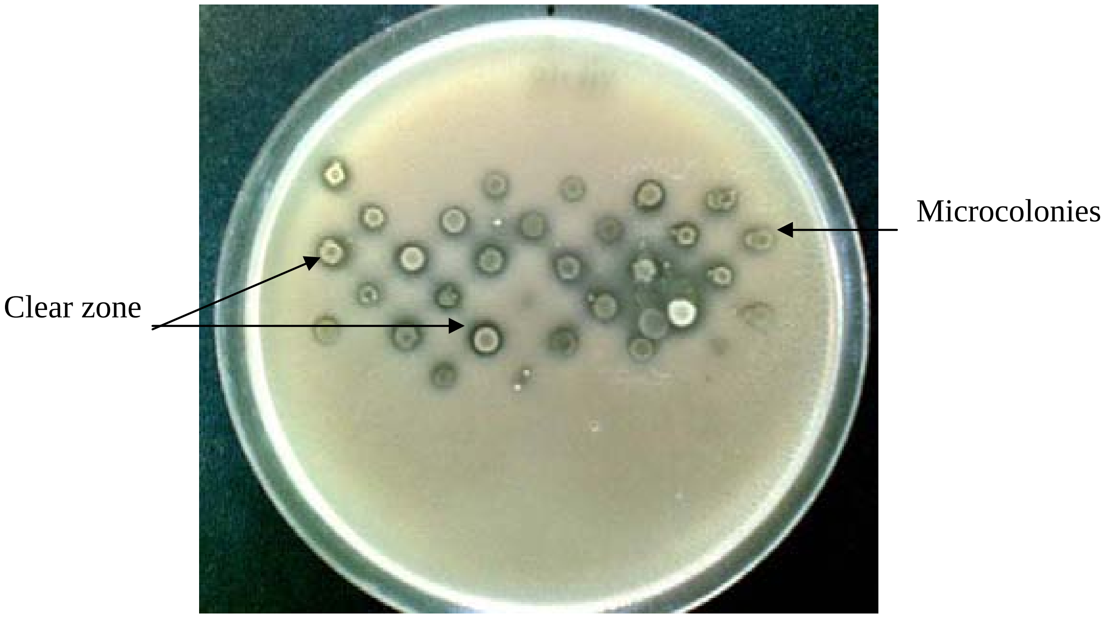


Figure 15. Antagonistic activity of *Bifidobacterium* against *Vibrio cholerae* with clear zone and microcolonies in 140 mm plate

Table 15. The summary antagonistic activity of *Lactobacillus* spp. against bacterial pathogens

Pathogens	Inhibition results (number of isolates)
ETEC	Inhibition (10): weak (1), microcolony (9)
EIEC	Inhibition (21): weak (6), microcolony (15)
EPEC	Inhibition (20): weak (4), microcolony (16)
EHEC	Inhibition (14): weak (6), microcolony (8)
S.Typhimurium	Inhibition (16): weak (5), microcolony (11)
E. coli	No inhibition
V. cholerae	Inhibition (30): strong (9), weak (19), microcolony (2)
S. flexneri	Inhibition (30): strong (6), weak (20), microcolony (4)
MRSA	Weak inhibition (6)
H. pylori	No inhibition

Table 16. The summary antagonistic activity of *Bifidobacterium* spp. against bacterial pathogens

Pathogens	Inhibition results (number of isolates)
ETEC	Inhibition as microcolony (5)
EIEC	Inhibition as microcolony (12)
EPEC	Inhibition as microcolony (4)
EHEC	Inhibition as microcolony (5)
S.Typhimurium	Inhibition as microcolony (13)
E. coli	No inhibition
V. cholerae	Inhibition (30): strong (10), weak (19), microcolony (1)
S. flexneri	Inhibition (28): strong (1), weak (21), microcolony (6)
MRSA	No inhibition
H. pylori	Inhibition (23): strong(5), weak (18)

Table 17. The summary antagonistic activity of *Streptococcus* spp. against bacterial pathogens

Pathogens	**Inhibition results (number of isolates)**
ETEC	No inhibition
EIEC	
EPEC	
EHEC	
S.Typhimurium	
E. coli	
V. cholerae	Inhibition (9): weak (8), microcolony (1)
S. flexneri	Partial in inhibition as microcolony (4)
MRSA	Weak inhibition of (5)
H. pylori	No inhibition

CHAPTER V

DISCUSSION

Breast milk is the best food for growing infant and has been shown to be a source of commensal and/or probiotic bacteria [2]. The commensal bacteria presented in breast milk were such as staphylococci, streptococci, lactobacilli, enterococci and bifidobacteria [3, 4]. These bacterial groups were shown to be associated with neonates gut microbiota and may also play role in the reduction of the incidence of infections in the breast-fed infant [2, 5]. Our results showed the diversity of lactobacilli, bifidobacteria and streptocooci in breast milk detected by the use of culture-dependent techniques and genotypic identification. MRS medium was a culture medium for the cultivation of *Lactobacillus* and *Bifidobacterium* [140]. However, our study demonstrated that only *Lactobacillus* was recovered on MRS agar. *Bifidobacterium* was detected only on MC agar which is Columbia medium modified by the addition of glucose, cysteine hydrochloride, agar and bromocresol purple for the differentiation of acid-producing as described by Beerens [68]. There are many type of media for detection of *Bifidobacterium* such as TPY medium (trypticase-phytone-yeast) described by Scardovi (1986) [141] and Columbia agar containing horse blood (5%, V/V) [140]. In fact, *Streptococcus* may grow on MRS medium but this work could not isolate these bacteria from this medium. *Streptococcus* isolates were detected on M 17 medium which is a selective media for lactic acid streptococci and recommended for the isolation of *S. thermophilus* from yogurt [142].

The skin swabs were cultured for *Lactobacillus*, *Bifidobacterium* and *Streptococcus* in the same condition as breast milk samples and none of them were recovered. This suggested that *Lactobacillus*, *Bifidobacterium* and *Streptococcus* found in breast milk were not from the contamination of these bacteria from nipple and the surrounding skin of volunteers. The origin of the live *Lactobacillus*, *Bifidobacterium* and *Streptococcus* in breast milk is still controversial. They may be from exogenous source such as the infant mouth and fecal of the mother [143] or the maternal gut involving maternal dendritic cells and macrophages [5, 144].

All isolates of *Lactobacillus, Bifidobacterium and Streptococcus* were genotypically identified by 16S rRNA gene sequencing. Forty *Lactobacillus* isolates were identified to 8 species such as *L. gasseri, L. salivarius, L. fermentum, L. mucosae, L. rhamnosus, L. casei, L. plantarum* and *L. oris*. These *Lactobacillus* isolates were recovered from 37 (36.27%) milk samples. The result demonstrated that the diversity of species was more than that found in previous studies but the number of positive samples was varied. Heikkila *et al*. isolated 7 *Lactobacillus* from 4 (10%) of 40 healthy lactating mothers in Finland. The majority of samples were taken within 90 days of delivery. These *Lactobacillus* species were *L. rhamnosus* and *L. crispatus* [3]. Martin *et al*. reported the isolation of three *Lactobacillus* species such as *L. gasseri* and *L. fermentum* from 8 (100%) lactating mothers [2, 145]. However, this study did not mention about the age of lactation.

Thirty-three *Bifidobacterium* isolates were identified to 5 species such as *B. longum, B. breve, B. psedocatenulatum, B. dentium* and *B. bifidum*. These *Bifidobacterium* isolates were recovered from 31 (30.39%) milk samples. The diversity of species was more but the number of positive samples was less than those of the previous study. Martin *et al*. was the first to report the isolation of bifidobacteria from 8 (34.78%) of 23 healthy lactating mothers in Spain. These *Bifidobacterium* species were *B. breve*, *B. adolescentis* and *B. bifidum* [4].

Twenty-six *Streptococcus* isolates were identified to 5 species such as *S. salivarius*, *S. lactarius, Streptococcus* sp., *S. mitis and S. parasanguis.* These *Streptococcus* isolates were recovered from in 17 (16.67%) milk samples. The diversity of species and the number of positive samples was less than those found in previous studies. Heikkila *et al*. isolated *Streptococcus* from 40 healthy lactating mothers in Finland. The result was shown 151 *Streptococcus* isolates and recovered from 29 (72.5%) milk samples. The *Streptococcus* species such as *S. salivarius, S. mitis, S. parasanguis, S. peroris, S. agalactiae* and *Streptococcus* sp. (oral) [3]. In fact, genus *Streptococcus* was the second most abundant in breast milk and associated with oral species. In this work *S. lactarius* which was reported as a novel species in breast milk [146] was also recovered from milk samples. *Staphylococcus* isolates which were picked up by technical errors from catalase test and positive with *Lactobacillus*-specific amplification revealed that genus-specific primers had homology with *Staphylococcus* DNA. Alignment of these primers with *S. aureus* and *S. epidermidis* 16S rRNA genes showed that they had 59.09% and 86.36%, respectively. It is therefore possible that the genus-specific primers could amplify *S. epidermidis* which is skin flora.

Polymerase chain reaction (PCR) method was used for detect of lactocbacilli, bifidobacteria and streptococci DNAs in breast milk. Targets DNA were extracted by Qiagen stool mini kit and add a bead-beading to the chemical lyses for increase cells lysis step. PCR amplicons were generated with genus-specific primers for *Lactobacillus*, *Bifidobacterium* and *Streptococcus*. The results demonstrated the presence of lactobacilli, bifidobacteria and streptococci DNAs in 94 (92.16%), 60 (58.82%) and 56 (54.90%) of 102 breast milk samples, respectively. Based on the false-positive result of genus-specific amplification of bacterial isolates in Table 9 , the positive result of DNA presence in breast milk samples was estimated to be 76.94% of *Lactobacillus*, 84% of *Bifidobacterium* and 100% of *Streptococcus*. Collado *et al*. reported the presence of *Lactobacillus, Bifidobacterium* and *Streptococcus* in 50 (100%) lactating mothers by quantitative real-time PCR [136]. In addition, Martin *et al*. reported the detection of *Bifidobacterium* in 22 (95.65%) of 23 milk samples by quantitative real-time PCR. They also showed that the percentage of *Bifidobacterium* DNA was ≤ 16% of total bacterial DNA [4]. Our result demonstrated that the number of bacterial isolates cultivated from breast milk samples was lower than that detected by PCR. This resulted from the fact that culture method is not perfect to recover all bacterial species in samples.

Since *Lactobacillus* and *Bifidobacterium* are anaerobic bacteria, they are sensitive to oxygen. Samples should be transported in anaerobic condition which was not feasible for liquid samples like breast milk and skin swab in peptone water. To acquire anaerobic condition, the lid of sample tube must be loosen resulting in sample leak. In addition, sample transportation was carried in cold which was not appropriate temperature for cold- sensitive *Streptococcus*. The result of *Streptococcus* prevalence in breast milk was then less than that of previous study [3].

Antagonistic activity assay was performed in all isolates of *Lactobacillus*, *Bifidobacterium* and *Streptococcus* against bacterial pathogens such as enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), *Salmonella* Typhimurium, *Shigella flexneri*, *Vibrio cholerae*, *Helicobacter pylori* and methicillin-resistant *Staphylococcus aureus* (MRSA). Thirteen *Lactobacillus* isolates such as Lac43 (*L. rhamnosus*), Lac44 (*L. casei*), Lac45 (*L. salivarius*), NL1 (*L. salivarius*), NL3 (*L. salivarius*), NL5 (*L. salivarius*), NL6 (*L. salivarius*), NL7 (*L. salivarius*), NL8 (*L. gasseri*), NL10 (*L. gasseri*), NL18 (*L. mucosae*), NL26 (*L. salivarius*) and NL50 (*L. fermentum*) had strong antagonistic activity against *V. cholerae* and *S. flexneri* and weak antagonistic activity against ETEC, EIEC, EPEC, EHEC and *S.* Typhimurium. Furthermore, six isolates of *Lactobacillus* such as Lac 40 (*L. salivarius*), Lac41 (*L. salivarius*), NL26 (*L. salivarius*), NL50 (*L. fermentum*), NL52 (*L. mucosae*) and NL53 (*L. fermentum*) could inhibit the growth of methicillin-resistant *Staphylococcus aureus* (MRSA). Since 2006, only Olivares *et al.* has reported breast - milk originated *Lactobacillus* such as *L. salivarius* CECT5713 and *L. gasseri* CECT5714 could inhibit the growth of *Salmonella cholerasuis*, *Escherichia coli*, *Staphylococcus aureus*, *Literia monocytogenes* and *Clostridium tyrobutyricum*, [32].Antagonistic activity was reported in *Lactobacillus* isolated from other sources such as four *Lactobacillus reuteri* strains could inhibit the growth of enteric pathogens (EHEC, ETEC, *Salmonella enterica*, *Shigella sonnei* and *Vibrio cholerae*) [11]. Parvathi *et al.* demonstrated that *Lactobacillus fermentum* isolated from the intestinal biopsy samples could inhibit the growth of enteric pathogens such as *E. coli*, *S. paratyphi*, and *S. sonnei* [147]. Raffaella *et al.* reported *Lactobacillus acidophilus* ATCC 4356 could inhibit growth of *Campylobacter jejuni* strains [148].

Eleven *Bifidobacterium* isolates such as Bif29 (*B. breve*), NB4 (*B. dentium*), NB11 (*B. dentium*), NB13 (*B. bifidum*), NB14 (*B. dentium*), NB15 (*B. bifidum*), NB16 (*B. bifidum*), NB17 (*B. longum*), NB28 (*B. longum*), NB31 (*B. breve*) and NB40 (*B. dentium*) had strong antagonistic activity against *V. cholerae* and *S. flexneri* and had partial antagonistic activity against ETEC, EIEC, EPEC, EHEC and *S.* Typhimurium as microcolony. In addition, five *Bifidobacterium* isolates including NB6 (*B. dentium*), NB8 (B. *longum*), NB14 (*B. dentium*), NB28 (*B. longum*) and NB31 (*B.*

breve) had strong antagonistic activity against *H. pylori*. Breast-milk originated *Bifidobacterium* spp. have not been reported for their antagonistic activity. Only *Bifidobacterium* isolated from feces were studied previously. Gibson *et al*. has shown that *B. infantis* isolated from infant feces could inhibit the growth of *E. coli* and *Clostridium perfringens* [149]. Bevilacqua *et al*. has shown bifidobacteria isolated from human feces could inhibit the growth of *Clostridium sporogenes* [150]. Collado *et al*. had demonstrated that *Bifidobacterium* isolated from feces could inhibit the growth of *Helicobacter pylori* [151].

Eight *Streptococcus* isolates such as St6 (*S. salivarius*), St12 (*S. salivarius*), St13 (*S. salivarius*), St14 (*S. salivarius*), St15 (*S. salivarius*), St 32 (*Streptococcus* sp.) and St34 (*S. salivarius*) had weak antagonistic activity against *V. cholerae*. Five isolates including St10 (*S. lactarius*), St11 (*S. lactarius*), NL4 (*Streptococcus* sp.), NL9 (*S. salivarius*) and St27 (*Streptococcus* sp.) weakly inhibited the growth of MRSA. There was only one report of Heikkila *et al*. demonstrating that *Streptococcus salivarius* isolated from breast milk had antagonistic activity against *Staphylococcus aureaus* [3].

Beneficial bacteria such as *Lactobacillus*, *Bifidobacterium* and *Streptococcus* isolated from breast milk had the ability to inhibit the growth of pathogenic bacteria. It has been documented that bacterial antagonistic activity may be from the production of acid, hydrogen peroxide or bacteriocin and small organic molecules [9, 145, 149-152]. Sigrid *et al*. reported the antibacterial activity of *Lactobacillus rhamnosus* GG against *Salmonella typhimurium* by the production of antimicrobial compound which was a low molecular weight, heat-stable, non-proteinaceous substance, thought to be lactic acid [153]. Collado *et al*. demonstrated that six *Bifidobacterium* isolated from feces could inhibit the growth of *H. pylori*. These antagonistic effects were found to relate to heat-stable, proteinaceous bactericidal substance, suspected to be antimicrobial peptides [151]. Cheikhyoussef *et al*. purified bacteriocin called bifidin I from *Bifidobacterium infantis* BCRC 14602 and demonstrated its ability to inhibit the growth of many Gram-positive and Gram-negative bacteria which cause food spoilage and food-borne diseases [76]. Mathot *et al.* reported that *S. thermophilus* could produce bacteriocin called thermophilins with the ability to inhibit the growth of *Clostridium tyrobutyricum* [13].

The mechanisms which breast milk-derived lactobacilli, bifidobacteria and streptococci employed to suppress the growth of pathogens in this study were not determined. They were of interest to investigate in further study.

CHAPTER VI

CONCLUSION

Culture and identification of beneficial bacteria from breast milk and swabs of nipple and surrounding skin of Thai healthy mothers were performed. It was found that 40 *Lactobacillus*, 33 *Bifidobacterium* and 26 *Streptococcus* isolates were recovered from 37 (36.27%), 31 (30.39%) and 17 (16.67%) samples, respectively. None of these bacteria was recovered from skin swabs. Isolated *Lactobacillus* spp. included *L. gasseri*, *L. salivarius*, *L. fermentum, L. mucosae, L. rhamnosus, L. casei, L. plantarum* and *L. oris* whereas isolated *Bifidobacterium* spp. were *B. longum, B. breve, B. pseudocatenulatum*, *B. dentium* and *B. bifidum.* Isolated *Streptococcus* was found to be *S. salivarius*, *S. lactarius*, *Streptococcus* sp., *S. mitis* and *S. parasangius*

Test for antagonistic activity against ETEC, EIEC, EPEC, EHEC and *S.* Typhimurium revealed that all isolates of *Lactobacillus*, *Bifidobacterium* and *Streptococcus* had weak, partial and no activity, respectively. Thirteen *Lactobacillus* isolates (Lac43, Lac44, Lac45, NL1, NL3, NL5, NL6, NL7, NL8, NL10, NL18, NL26 and NL50) and 11 *Bifidobacterium* isolates (Bif29, NB4, NB11, NB13, NB14, NB15, NB16, NB17, NB28, NB31 and NB40) demonstrated strong antagonistic activity against *V. cholerae* and *S. flexneri*. Furthermore, six *Lactobacillus* isolates (Lac 40, Lac41, NL26 NL50, NL52 and NL53) and 5 *Streptococcus* isolates (St10, St11, NL4, NL9 and St27) weakly inhibited the growth of MRSA. In addition, five *Bifidobacterium* isolates (NB6, NB8, NB14, NB28 and NB31) had strong antagonistic activity against *H. pylori*.

The majority of lactobacilli, bifidobacteria and streptococci that had strong inhibitory activities against bacterial pathogens belonged to *L. salivarius, L. gasseri, L. mucosae, L. fermentum*. *B. dentium, B. bifidum*. Since specific strains of *Lactobacillus*, *Bifidobacterium* and *Streptococcus* had probiotic properties, these breast milk-derived bacteria were probiotic candidates for further study to elucidate their antagonistic mechanism against gastrointestinal bacterial pathogens.

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APPENDICES

APPENDIX A

MATERIALS AND EQUIPMENTS

Materials and reagents

- Agarose (Research organism, USA)
- Anaerobic indicator (Oxoid, Basingstroke, Hamps, UK)
- Boric acid (Sigma, USA)
- Brain heart infusion agar (Difco, USA)
- Brain heart infusion broth (Difco, USA)
- Columbia blood agar base (Oxoid, Basingstroke, Hamps, UK)
- Cysteine hydrochloride (Sigma, USA)
- Dextrose bacteriological (Oxoid, Basingstroke, Hamps, UK)
- Ethylene diamine tetraacetic acid (EDTA) (Sigma, USA)
- Ethidium bromide (Bio Rad, USA)
- Gaspak (AnaeroPack-Anaero, Mitsubishi, Japan)
- GeneRuler^{TM} 100bp DNA Ladder Plus (Fermentas, USA)
- Glycerol (Merck, Germany)
- MRS agar (Oxoid, Basingstroke, Hamps, UK)
- MRS broth (Oxoid, Basingstroke, Hamps, UK)
- M17 agar (Oxoid, Basingstroke, Hamps, UK)
- M17 broth (Oxoid, Basingstroke, Hamps, UK)
- Peptone bacteriological (Oxoid, Basingstroke, Hamps, UK)
- Proteinase K (Sigma, USA)

- QIAamp® DNA Stool Mini Kit (Qiagen, Hilden, Germany)
- QIAquick PCR Purification Kit (Qiagen, Hilden, Germany)
- Sodium chloride (NaCl) (Sigma, USA)
- Tris base (Sigma, USA)
- Tween 20 (Merck, Germany)
- Tween 80 (Sigma, USA)
- Yeast extract (Difco, USA)

2. Equipments

- Anaerobic Chamber (Concept Plus, Ruskinn Technology, UK)
- Anaerobic Jar (BBL, USA)
- Autoclave (Hirayama, Japan)
- Autopipettes (Gilson, France)
- Centrifuge (Kubota, Japan)
- Deep Freezer (-20^{0}C) (Sanyo, Japan)
- Deep Freezer (-80^{0}C) (Sanyo, Japan)
- Electrophoresis chamber (BioRad, USA)
- Frogger (DAN-KAR CCRP, USA)
- Gel doc (BioRad, USA)
- Heat block (Scientific, USA)
- Hot air oven (Haraeus, Germany)
- Incubator (Forma Scientific, USA)
- Light Microscope (Nikon, Japan)
- Microcentrifuge (Eppendorf, USA)

- pH meter (Orion, USA)
- Thermal cycler (Eppendorf, Hamburg, Germany)
- Vortex mixer (Scientific, USA)
- Water bath (Memmert, USA)

3. Software and program

- GenBank DNA database search (http://www.ncbi.nlm.gov/BLAST).
- Multalin program (http://bioinfo.genotoul.fr/multalin)
- Sequence mach program of the Ribosomal Database Project II (RDP-II; http://rdp.cme.msu.edu)

APPENDIX B

PREPARATION OF MEDIA AND REAGENT

Media for lactobacilli

1. MRS agar

MRS agar (oxoid)	62	g
Distilled water	1,000	ml

2. MRS broth

MRS broth (oxoid)	52	g
Distilled water	1,000	ml

3. 20% glycerol MRS broth

Glycerol	20	ml
Distilled water	40	ml
MRS broth	40	ml

(MRS 2.08 g + DW 40 ml)

20% glycerol MRS broth using for kept lactobacilli cell in deep freeze.

Media for bifidobacteria

4. Modified Columbia (MC) medium

Columbia agar base (oxoid)	39	g
Glucose	5	g
Cysteine hydrochloride	0.5	g
Agar	5	g
Distilled water	1,000	ml

The pH was adjusted to 7.3 before autoclaving at 121^0C for 15 minutes.

When use the media as differential medium add 0.03 g/l of bromocresol purple indicator for observed glucose fermentation.

5. Brain Heart Infusion Broth (BHB)

Brain Heart Infusion (BBL)	37	g
Yeast extract	5	g
Cysteine hydrochloride	0.5	g
Distilled water	1,000	ml

The pH was adjusted to 7.2 before autoclaving at 121^{0}C for 15 minutes. Brain heart infusion broth using as enrichment medium.

6. Brain Heart Infusion Agar (BHA)

Brain Heart Infusion Agar (BBL)	52	g
Yeast extract	5	g
Cysteine hydrochloride	0.5	g
Distilled water	1,000	ml

Brain heart infusion agar using for antagonistic activity assay.

7. 20% glycerol Brain Heart Infusion Broth

Brain Heart Infusion (BBL)	3.7	g
Yeast extract	0.5	g
Cysteine hydrochloride	0.05	g
Glycerol	20	ml
Distilled water	80	ml

20% glycerol BHB using for kept bifidobacteria cell in deep freeze.

Media for streptococci

8. M17 agar

M17 agar (oxoid)	48.25	g
Distilled water	950	ml
10% lactose solution	50	ml

9. 20% glycerol M17 broth

Glycerol	20	ml
Distilled water	40	ml
M17 broth (oxoid)	40	ml

(M 17 1.37 g+ Lactose solution 5 ml + DW 35 ml)

Reagent for molecular analysis

10. 0.5M EDTA, pH 8.0

Ethylene diamine tetraacetic acid (EDTA)	93.05	g
Distilled water	500	ml

Dissolve 93.05 g of EDTA in 400 ml of distilled water, then the pH was adjusted to 8.0 with NaOH (pellets) and final volume was bought up to 500 ml. The stock reagent sterile by autoclaving at 121^{0}C at 15 pounds/inch^2 pressure for 15 minutes. The solution was stored at room temperature.

11. 5X TBE

Tris base	54	g
Boric acid	27.5	g
0.5M EDTA pH 8.0	20	ml
Distilled water	1,000	ml

Dissolve all of ingradients in 1,000 ml of distilled water. The stock reagent sterile by autoclaving at 121^{0}C at 15 pounds/inch^2 pressure for 15 minutes. The solution was stored at room temperature.

12. 1M Tris-HCl, pH 8.0

Tris base	121.1	g
Distilled water	1,000	ml

Dissolve 121.1 g of Tris base in 800 ml of distilled water. Adjust the pH to the desired value by adding concentrated HCl 42 ml and allow the solution to cool to room temperature before making final adjustments to the pH 8.0. Adjust the volume of the solution to 1 liter with distilled water. Dispense in to aliquots and sterilize by autoclaving.

13. 10X Digestion buffer

The stock reagent 10X digestion buffer contained 5% tween 20 and 10 mg/ml proteinase K in 0.2 M Tris pH 8.3. For example prepare 4 ml of the stock reagent.

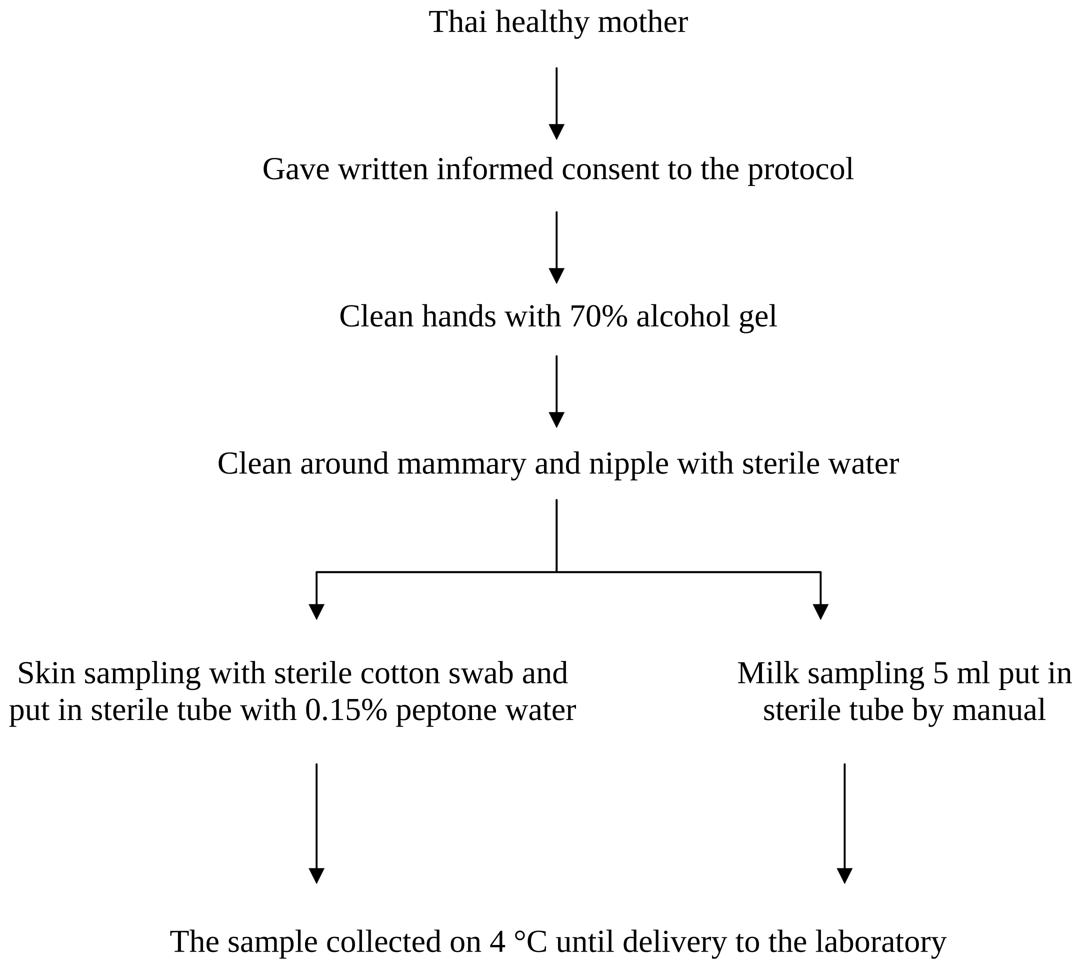
Tween 20	0.2	ml
Proteinase K	40	mg
1M Tris pH 8.3	0.8	ml
Distilled water	3.0	ml

Dissolve 40 mg of Proteinase K in 3 ml of distilled water adding Tween 20 and 1M Tris pH 8.3 making final volume to 4 ml. Mix well and store at 4^0C.

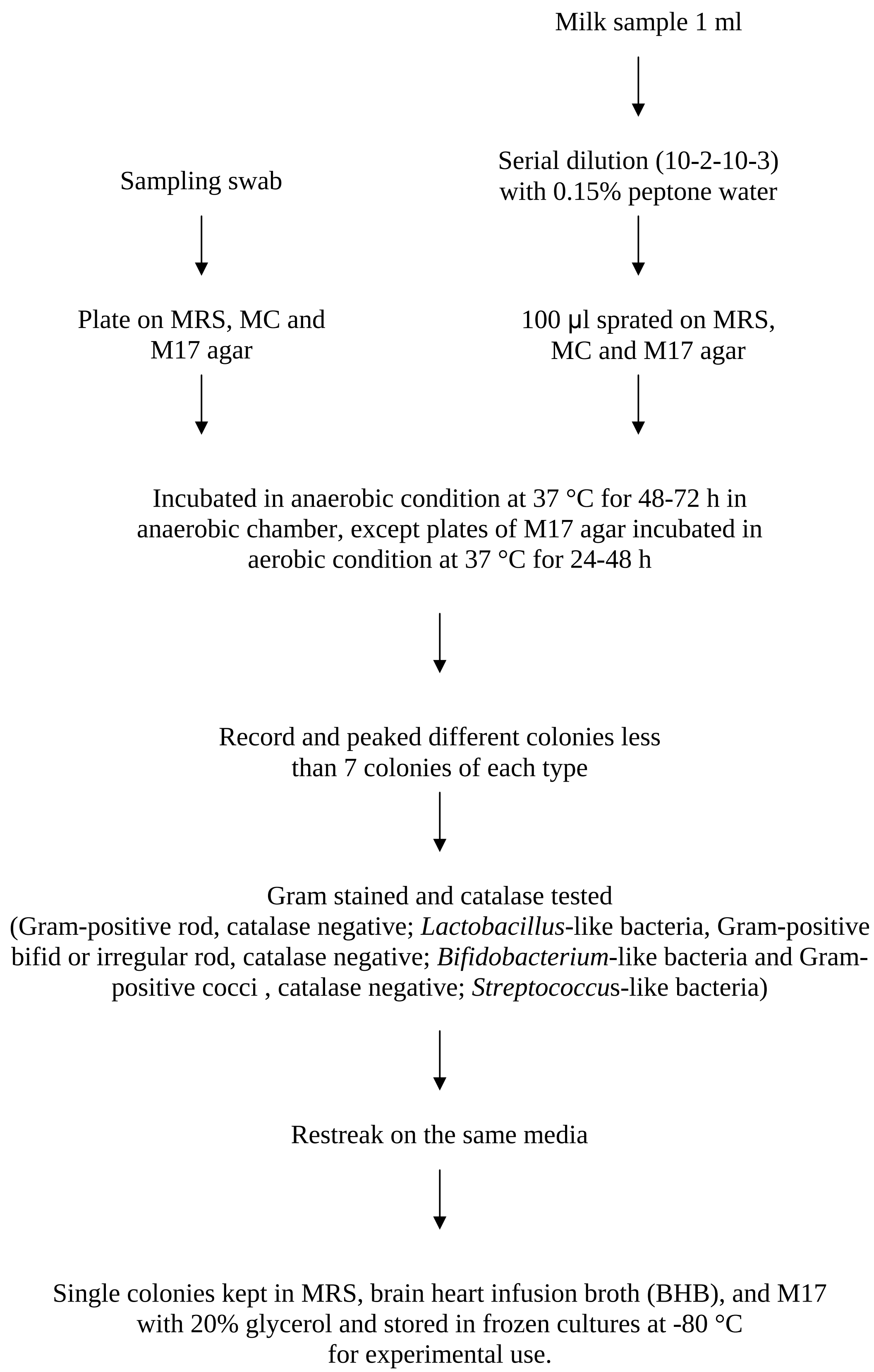
APPENDIX C

FLOW CHART OF PROTOCAL

1. Collection of breast milk



2. Isolation of *Lactobacillus*, *Bifidobacterium* and *Streptococcus* from breast milk



Milk sample 1 ml

Serial dilution (10-2-10-3) with 0.15% peptone water

Sampling swab

Plate on MRS, MC and M17 agar

100 μl sprated on MRS, MC and M17 agar

Incubated in anaerobic condition at 37 °C for 48-72 h in anaerobic chamber, except plates of M17 agar incubated in aerobic condition at 37 °C for 24-48 h

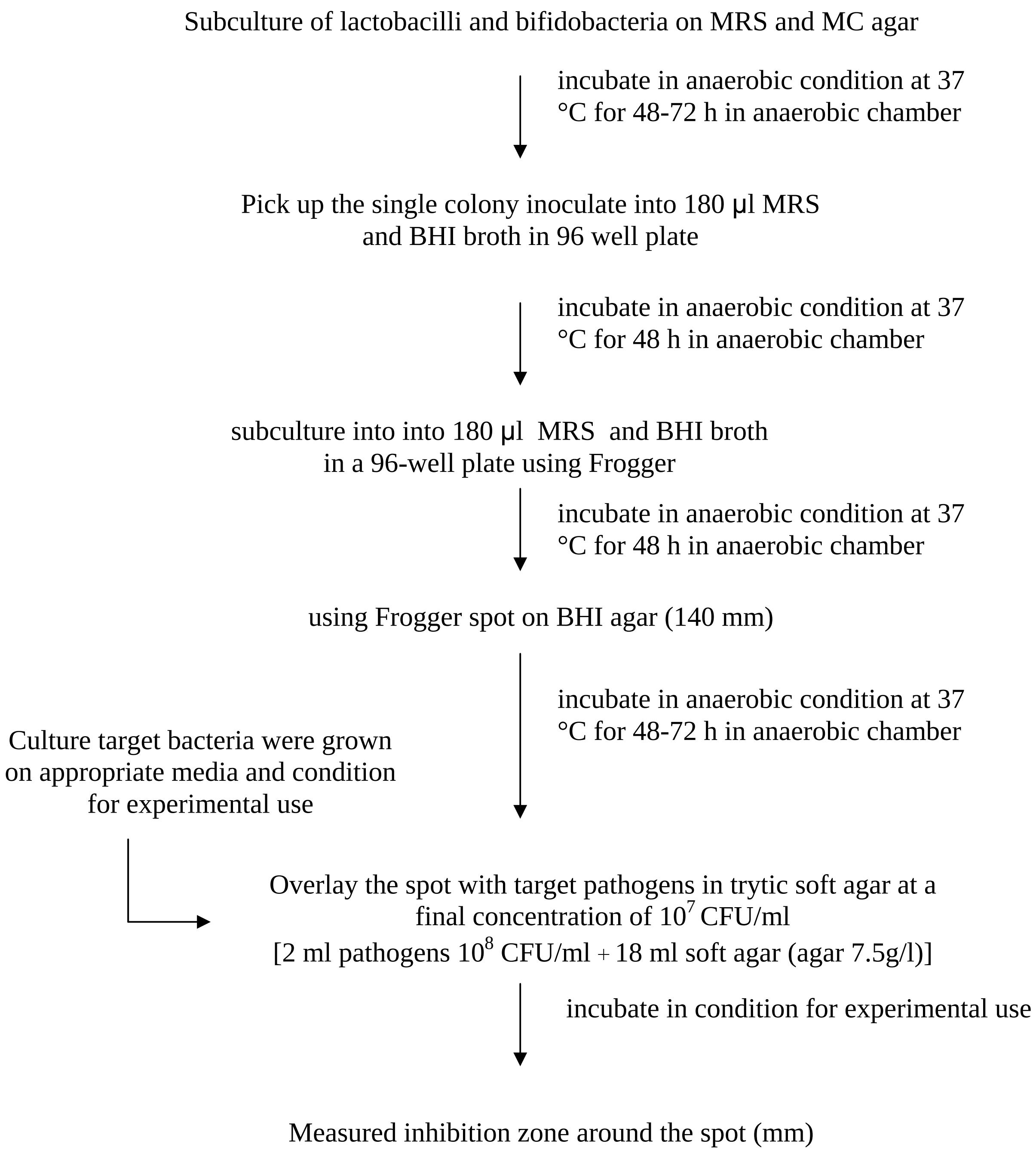
Record and peaked different colonies less than 7 colonies of each type

Gram stained and catalase tested (Gram-positive rod, catalase negative; *Lactobacillus*-like bacteria, Gram-positive bifid or irregular rod, catalase negative; *Bifidobacterium*-like bacteria and Gram-positive cocci , catalase negative; *Streptococcus*-like bacteria)

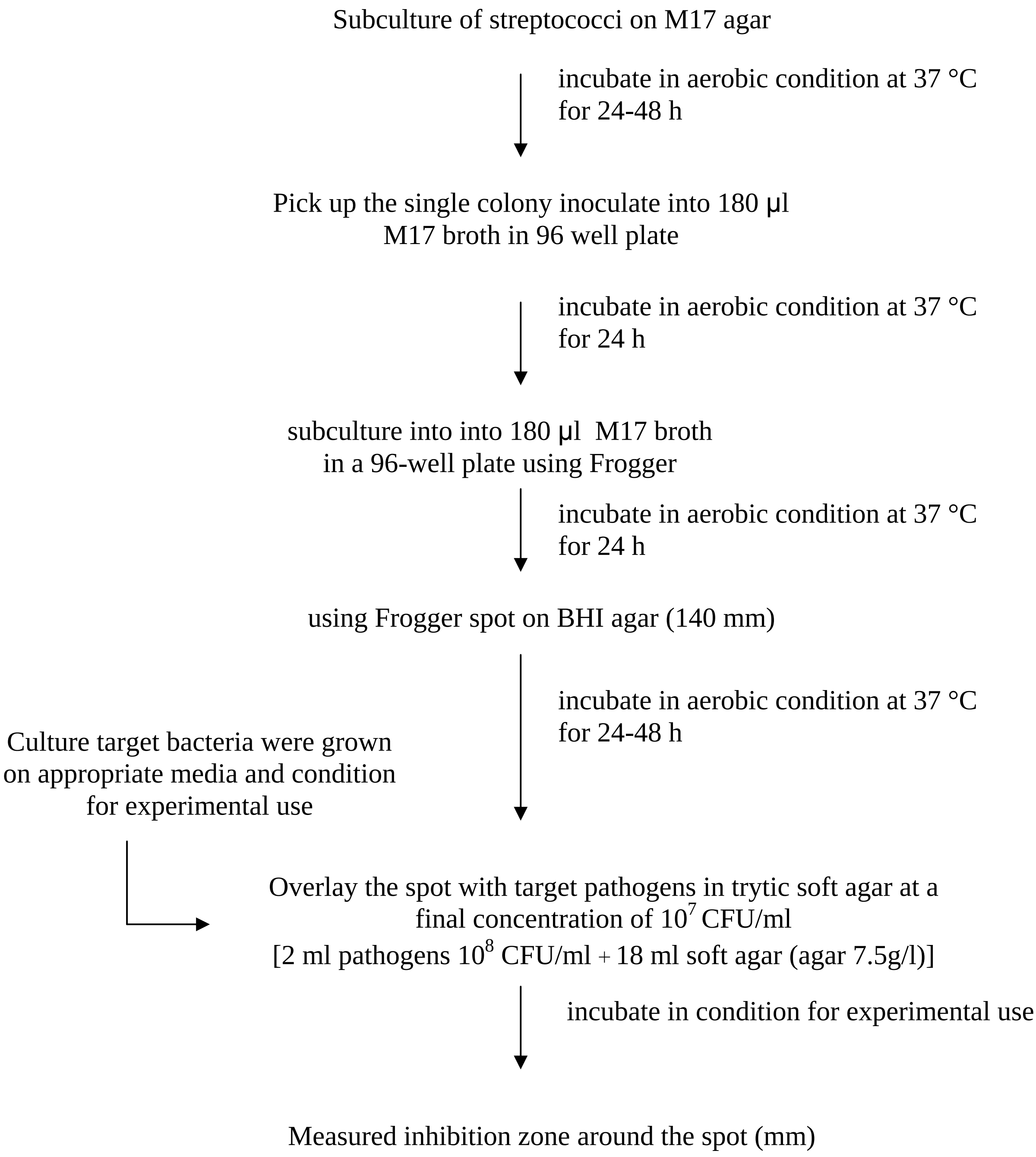
Restreak on the same media

Single colonies kept in MRS, brain heart infusion broth (BHB), and M17 with 20% glycerol and stored in frozen cultures at -80 °C for experimental use.

3. Antagonistic activity assay of lactobacilli and bifidobacteria using agar spot method



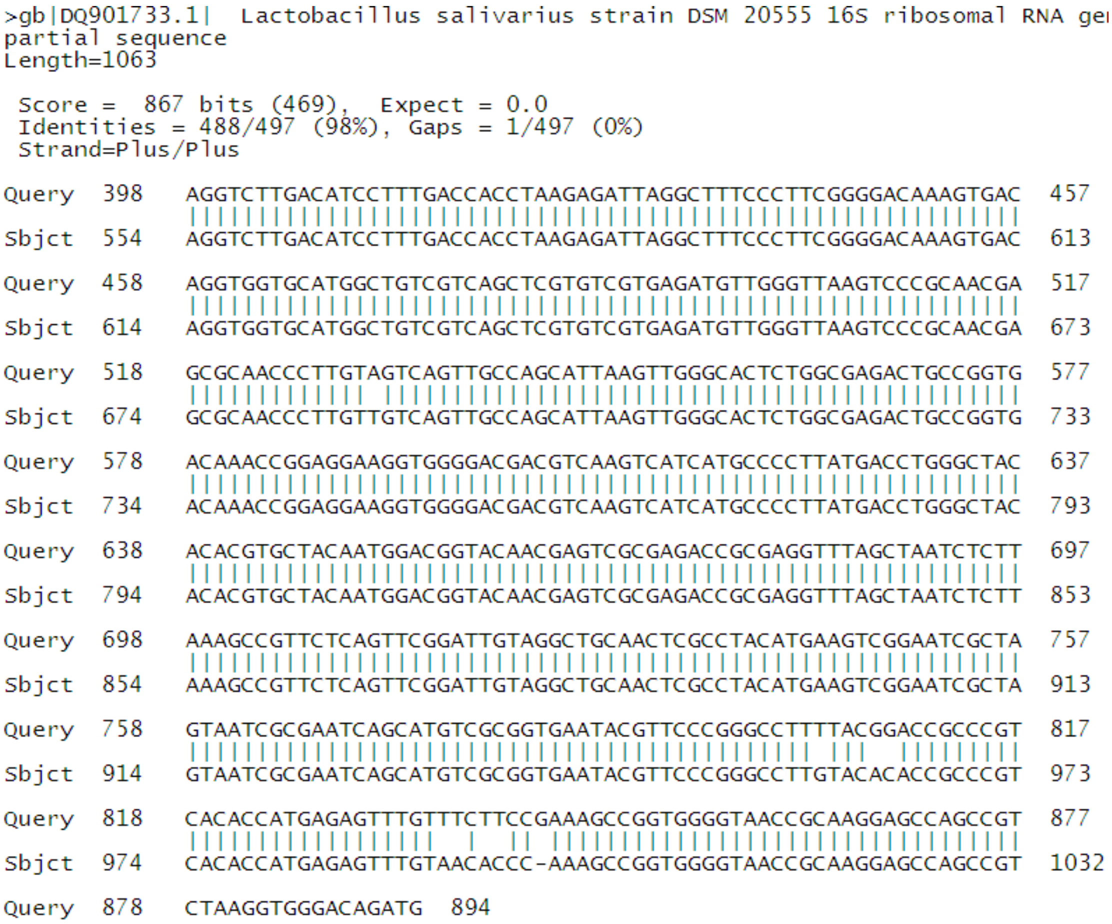
4. Antagonistic activity assay of streptococci using agar spot method



APPENDIX D

NECLEOTIDE ALINGMENT

1. The similarly alignment 16S rRNA gene sequence of *Lactobacillus* spp. isolated from breast milk with the 16S rRNA gene sequence of *Lactobacillus* spp. published in NCBI data bank.



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partial sequence
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 Identities = 488/497 (98%), Gaps = 1/497 (0%)
 Strand=Plus/Plus

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Query  518    GCGCAACCCTTGTAGTCAGTTGCCAGCATTAAGTTGGGCACTCTGGCGAGACTGCCGGTG  577
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Sbjct  674    GCGCAACCCTTGTTGTCAGTTGCCAGCATTAAGTTGGGCACTCTGGCGAGACTGCCGGTG  733

Query  578    ACAAACCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGACCTGGGCTAC  637
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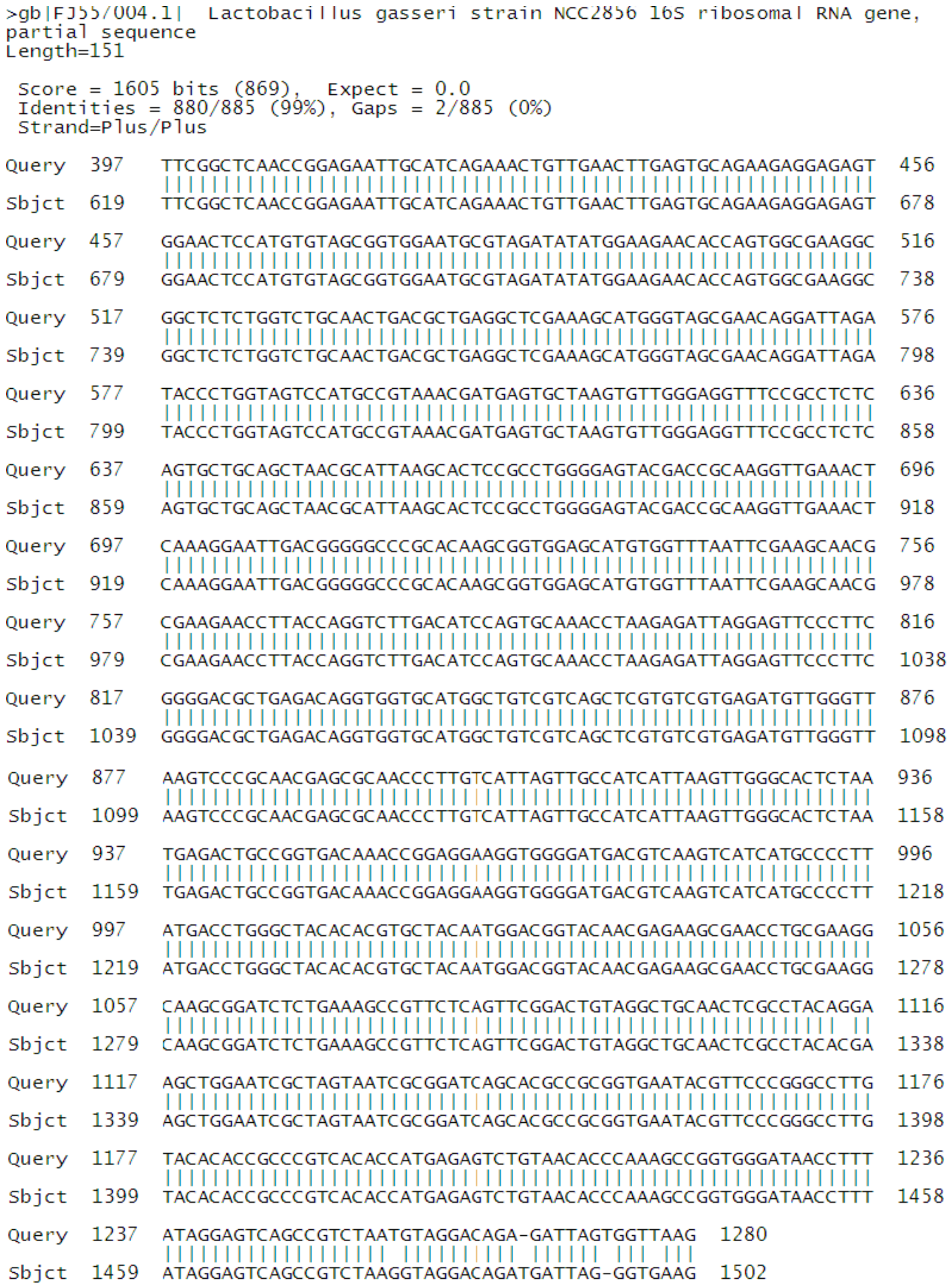

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Length=151

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 Identities = 880/885 (99%), Gaps = 2/885 (0%)
 Strand=Plus/Plus

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Sbjct  679   GGAACTCCATGTGTAGCGGTGGAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGC  738

Query  517   GGCTCTCTGGTCTGCAACTGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGA  576
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Sbjct  739   GGCTCTCTGGTCTGCAACTGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGA  798

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Sbjct  799   TACCCTGGTAGTCCATGCCGTAAACGATGAGTGCTAAGTGTTGGGAGGTTTCCGCCTCTC  858

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Sbjct  1099  AAGTCCCGCAACGAGCGCAACCCTTGTCATTAGTTGCCATCATTAAGTTGGGCACTCTAA  1158

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Length=1568

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 Identities = 1477/1495 (99%), Gaps = 14/1495 (1%)
 Strand=Plus/Plus

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Sbjct  104   TGGTTTACCAGCGAGTGGCGGACGGGTGAGTAACACGTAGGTAACCTGCCCCAAAGCGGG  163

Query  128   GGATAACATTTGGAAACAGATGCTAATACCGCATAACAATTTGAATCGCATGATTCAAAT  187
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Sbjct  164   GGATAACATTTGGAAACAGATGCTAATACCGCATAACAATTTGAATCGCATGATTCAAAT  223

Query  188   TTAAAAGATGGCTTCGGCTATCACTTTGGGATGGACCTGCGGCGCATTAGCTTGTTGGTA  247
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Sbjct  821   -CCCTGGTAG-TCCATG-CCGT-AAACGATG-AGTGCTAGGTGTTGG-A-GGGTTTCCG-  872

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Query  908   TGAAACTCAAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCG  967
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Sbjct  932   TGAAACTC-AAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCG  990

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Sbjct  991   AAGCTACGCGAAGAACCTTACCAGGTCTTGACATCTTGCGCCAACCCTAGAGATAGGGCG  1050

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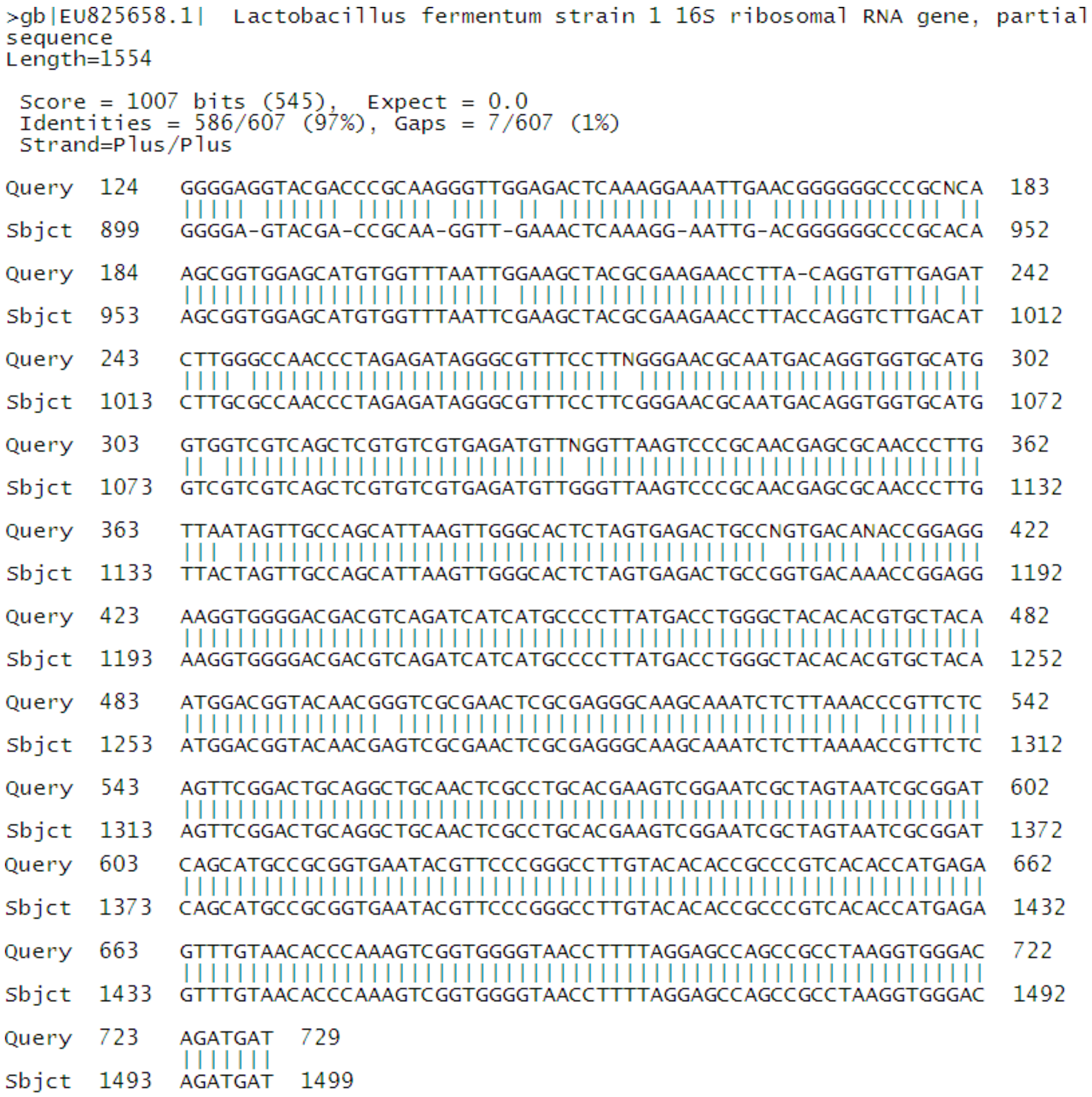

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Length=1554

 Score = 1007 bits (545),  Expect = 0.0
 Identities = 586/607 (97%), Gaps = 7/607 (1%)
 Strand=Plus/Plus

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Sbjct  899   GGGGA-GTACGA-CCGCAA-GGTT-GAAACTCAAAGG-AATTG-ACGGGGGGCCCGCACA  952

Query  184   AGCGGTGGAGCATGTGGTTTAATTGGAAGCTACGCGAAGAACCTTA-CAGGTGTTGAGAT  242
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Query  423   AAGGTGGGGACGACGTCAGATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACA  482
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Sbjct  1193  AAGGTGGGGACGACGTCAGATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACA  1252

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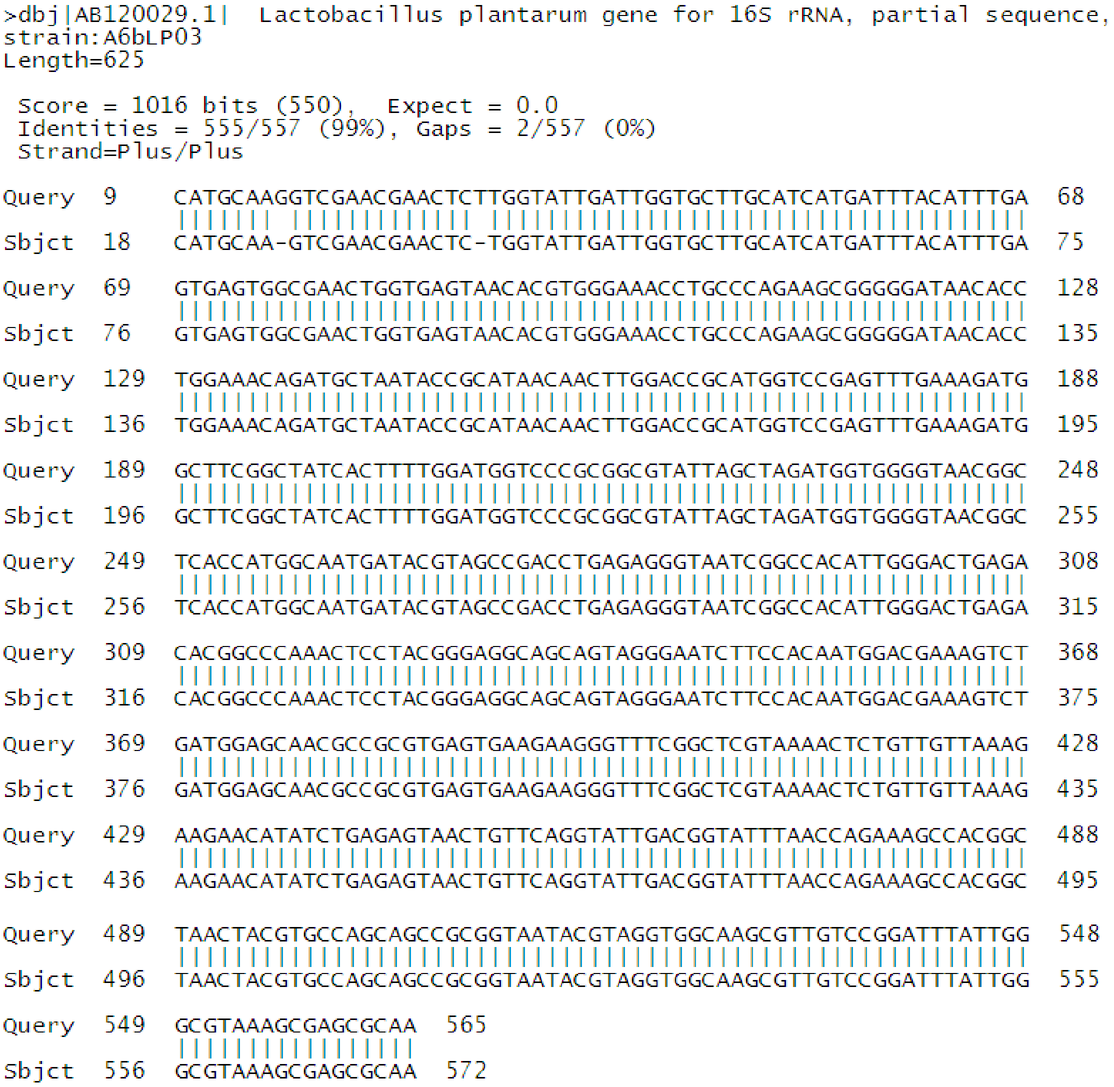

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strain:A6bLP03
Length=625

 Score = 1016 bits (550),  Expect = 0.0
 Identities = 555/557 (99%), Gaps = 2/557 (0%)
 Strand=Plus/Plus

Query  9    CATGCAAGGTCGAACGAACTCTTGGTATTGATTGGTGCTTGCATCATGATTTACATTTGA  68
            ||||||| ||||||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct  18   CATGCAA-GTCGAACGAACTC-TGGTATTGATTGGTGCTTGCATCATGATTTACATTTGA  75

Query  69   GTGAGTGGCGAACTGGTGAGTAACACGTGGGAAACCTGCCCAGAAGCGGGGGATAACACC  128
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  76   GTGAGTGGCGAACTGGTGAGTAACACGTGGGAAACCTGCCCAGAAGCGGGGGATAACACC  135

Query  129  TGGAAACAGATGCTAATACCGCATAACAACTTGGACCGCATGGTCCGAGTTTGAAAGATG  188
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  136  TGGAAACAGATGCTAATACCGCATAACAACTTGGACCGCATGGTCCGAGTTTGAAAGATG  195

Query  189  GCTTCGGCTATCACTTTTGGATGGTCCCGCGGCGTATTAGCTAGATGGTGGGGTAACGGC  248
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  196  GCTTCGGCTATCACTTTTGGATGGTCCCGCGGCGTATTAGCTAGATGGTGGGGTAACGGC  255

Query  249  TCACCATGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGA  308
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  256  TCACCATGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGA  315

Query  309  CACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCT  368
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  316  CACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCT  375

Query  369  GATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAG  428
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  376  GATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAG  435

Query  429  AAGAACATATCTGAGAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGC  488
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  436  AAGAACATATCTGAGAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGC  495

Query  489  TAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGG  548
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  496  TAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGG  555

Query  549  GCGTAAAGCGAGCGCAA  565
            |||||||||||||||||
Sbjct  556  GCGTAAAGCGAGCGCAA  572
```

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>gb|JQ412726.1|  Lactobacillus casei strain L8 16S ribosomal RNA gene, partial
sequence
Length=1518

 Score =  673 bits (364),  Expect = 0.0
 Identities = 369/371 (99%), Gaps = 2/371 (1%)
 Strand=Plus/Plus

Query  7    CTATACATGCCAAGTCGGAACGAGTTCTCGTTGATGATCGGTGCTTGCACCGAGATTCAA  66
            ||||||||| |||||| |||||||||||||||||||||||||||||||||||||||||||
Sbjct  15   CTATACATG-CAAGTC-GAACGAGTTCTCGTTGATGATCGGTGCTTGCACCGAGATTCAA  72

Query  67   CATGGAACGAGTGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCTTAAGTGGGGGAT  126
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  73   CATGGAACGAGTGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCTTAAGTGGGGGAT  132

Query  127  AACATTTGGAAACAGATGCTAATACCGCATAGATCCAAGAACCGCATGGTTCTTGGCTGA  186
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  133  AACATTTGGAAACAGATGCTAATACCGCATAGATCCAAGAACCGCATGGTTCTTGGCTGA  192

Query  187  AAGATGGCGTAAGCTATCGCTTTTGGATGGACCCGCGGCGTATTAGCTAGTTGGTGAGGT  246
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  193  AAGATGGCGTAAGCTATCGCTTTTGGATGGACCCGCGGCGTATTAGCTAGTTGGTGAGGT  252

Query  247  AATGGCTCACCAAGGCGATGATACGTAGCCGAACTGAGAGGTTGATCGGCCACATTGGGA  306
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  253  AATGGCTCACCAAGGCGATGATACGTAGCCGAACTGAGAGGTTGATCGGCCACATTGGGA  312

Query  307  CTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGC  366
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  313  CTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGC  372

Query  367  AAGTCTGATGG  377
            |||||||||||
Sbjct  373  AAGTCTGATGG  383
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>gb|HQ697661.1|  Lactobacillus oris strain 47-219 16S ribosomal RNA gene, partial
sequence
Length=567

 Score =  248 bits (134),  Expect = 1e-62
 Identities = 152/163 (93%), Gaps = 0/163 (0%)
 Strand=Plus/Plus

Query  21   AGCGCACTGNNCCAACAGAAATGACGTGCTTGCACTGATTTGACGTTGNATTCCCAGTGA  80
            |||||||||  ||||||||||||||||||||||||||||||||||||| |||||||||||
Sbjct  63   AGCGCACTGGCCCAACAGAAATGACGTGCTTGCACTGATTTGACGTTGGATTCCCAGTGA  122

Query  81   GCGGCGGACGGGTGAGTANCACGTGGCCCACCTGCCCCAAAGCGGGGGATAACATTTGGA  140
            |||||||||||||||||| ||||||| | |||||||||||||||||||||||||||||||
Sbjct  123  GCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCCCAAAGCGGGGGATAACATTTGGA  182

Query  141  AACAGGTGCTAATACCGCATAATTTGGAATACTATATGttttt  183
            |||||||||||||||||||||| |||||| || | ||| ||||
Sbjct  183  AACAGGTGCTAATACCGCATAACTTGGAAAACCACATGGTTTT  225
```

```
>gb|HM218396.1|  Lactobacillus rhamnosus strain NM94-5 16S ribosomal RNA gene,
partial sequence
Length=1504

 Score = 2623 bits (1420),  Expect = 0.0
 Identities = 1469/1491 (99%), Gaps = 16/1491 (1%)
 Strand=Plus/Plus

Query  10    ATACATGCAAGTCGAACGAGTTCTGATTATTGAAAGGTGCTTGCATCTTGATTTAATTTT  69
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  28    ATACATGCAAGTCGAACGAGTTCTGATTATTGAAAGGTGCTTGCATCTTGATTTAATTTT  87

Query  70    GAACGAGTGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCTTAAGTGGGGGATAACA  129
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  88    GAACGAGTGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCTTAAGTGGGGGATAACA  147

Query  130   TTTGGAAACAGATGCTAATACCGCATAAATCCAAGAACCGCATGGTTCTTGGCTGAAAGA  189
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  148   TTTGGAAACAGATGCTAATACCGCATAAATCCAAGAACCGCATGGTTCTTGGCTGAAAGA  207

Query  190   TGGCGTAAGCTATCGCTTTTGGATGGACCCGCGGCGTATTAGCTAGTTGGTGAGGTAACG  249
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  208   TGGCGTAAGCTATCGCTTTTGGATGGACCCGCGGCGTATTAGCTAGTTGGTGAGGTAACG  267

Query  250   GCTCACCAAGGCAATGATACGTAGCCGAACTGAGAGGTTGATCGGCCACATTGGGACTGA  309
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  268   GCTCACCAAGGCAATGATACGTAGCCGAACTGAGAGGTTGATCGGCCACATTGGGACTGA  327

Query  310   GACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAGT  369
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  328   GACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAGT  387

Query  370   CTGATGGAGCAACGCCGCGTGAGTGAAGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTGG  429
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  388   CTGATGGAGCAACGCCGCGTGAGTGAAGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTGG  447

Query  430   AGAAGAATGGTCGGCAGAGTAACTGTTGTCGGCGTGACGGTATCCAACCAGAAAGCCACG  489
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  448   AGAAGAATGGTCGGCAGAGTAACTGTTGTCGGCGTGACGGTATCCAACCAGAAAGCCACG  507

Query  490   GCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTTATT  549
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  508   GCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTTATT  567

Query  550   GGGCGTAAAGCGAGCGCAGGCGGTTTTTNTTAAGTCCTGANGTGAAAANNCCNTCGGGCT  609
             |||||||||||||||||||||||||||| | ||||| ||| ||||||   || |||| ||
Sbjct  568   GGGCGTAAAGCGAGCGCAGGCGGTTTTT-T-AAGTC-TGATGTGAAAGC-CC-TCGG-CT  621

Query  610   TTNNCCCGAGGGAAAGTGCATCCGGAAACTGGGAAAACTTTGAGTGCAGAAGNAGGACAG  669
             |   || |||| || ||||||| ||||||||||||| ||| ||||||||||| |||||||
Sbjct  622   TAA-CC-GAGG-AA-GTGCATC-GGAAACTGGGAAA-CTT-GAGTGCAGAAG-AGGACAG  673

Query  670   TGGAAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAG  729
             ||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  674   TGGAA-CTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAG  732

Query  730   GCGGCTGTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTA  789
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  733   GCGGCTGTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTA  792

Query  790   GATACCCCTGGTAGTCCATGCCGTAAACGATGAATGCTAGGTGTTGGAGGGTTTCCGCCC  849
             ||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  793   GATACCC-TGGTAGTCCATGCCGTAAACGATGAATGCTAGGTGTTGGAGGGTTTCCGCCC  851

Query  850   TTCAGTGCCGCAGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGACCGCAAGGTTGAA  909
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  852   TTCAGTGCCGCAGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGACCGCAAGGTTGAA  911

Query  910   ACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCA  969
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  912   ACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCA  971
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Query  970   ACGCGAAGAACCTTACCAGGTCTTGACATCTTTTGATCACCTGAGAGATCAGGTTTCCCC  1029
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  972   ACGCGAAGAACCTTACCAGGTCTTGACATCTTTTGATCACCTGAGAGATCAGGTTTCCCC  1031

Query  1030  TTCGGGGGCAAAATGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGG  1089
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1032  TTCGGGGGCAAAATGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGG  1091

Query  1090  GTTAAGTCCCGCAACGAGCGCAACCCTTATGACTAGTTGCCAGCATTTAGTTGGGCACTC  1149
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1092  GTTAAGTCCCGCAACGAGCGCAACCCTTATGACTAGTTGCCAGCATTTAGTTGGGCACTC  1151

Query  1150  TAGTAAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCC  1209
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1152  TAGTAAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCC  1211

Query  1210  CTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAACGAGTTGCGAGACCGCGA  1269
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1212  CTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAACGAGTTGCGAGACCGCGA  1271

Query  1270  GGTCAAGCTAATCTCTTAAAGCCATTCTCAGTTCGGACTGTAGGCTGCAACTCGCCTACA  1329
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1272  GGTCAAGCTAATCTCTTAAAGCCATTCTCAGTTCGGACTGTAGGCTGCAACTCGCCTACA  1331

Query  1330  CGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCC  1389
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1332  CGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCC  1391

Query  1390  TTGTACACACCGCCCGTCACACCATGAGAGTTTGTAACACCCGAAGCCGGTGGCGTAACC  1449
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1392  TTGTACACACCGCCCGTCACACCATGAGAGTTTGTAACACCCGAAGCCGGTGGCGTAACC  1451

Query  1450  CTTTTAGGGAGCGAGCCGTCTAAGGTGGGACAAATGATTAGTGTGAAGTCG  1500
             ||||||||||||||||||||||||||||||||||||||||| |||||||||
Sbjct  1452  CTTTTAGGGAGCGAGCCGTCTAAGGTGGGACAAATGATTAGGGTGAAGTCG  1502
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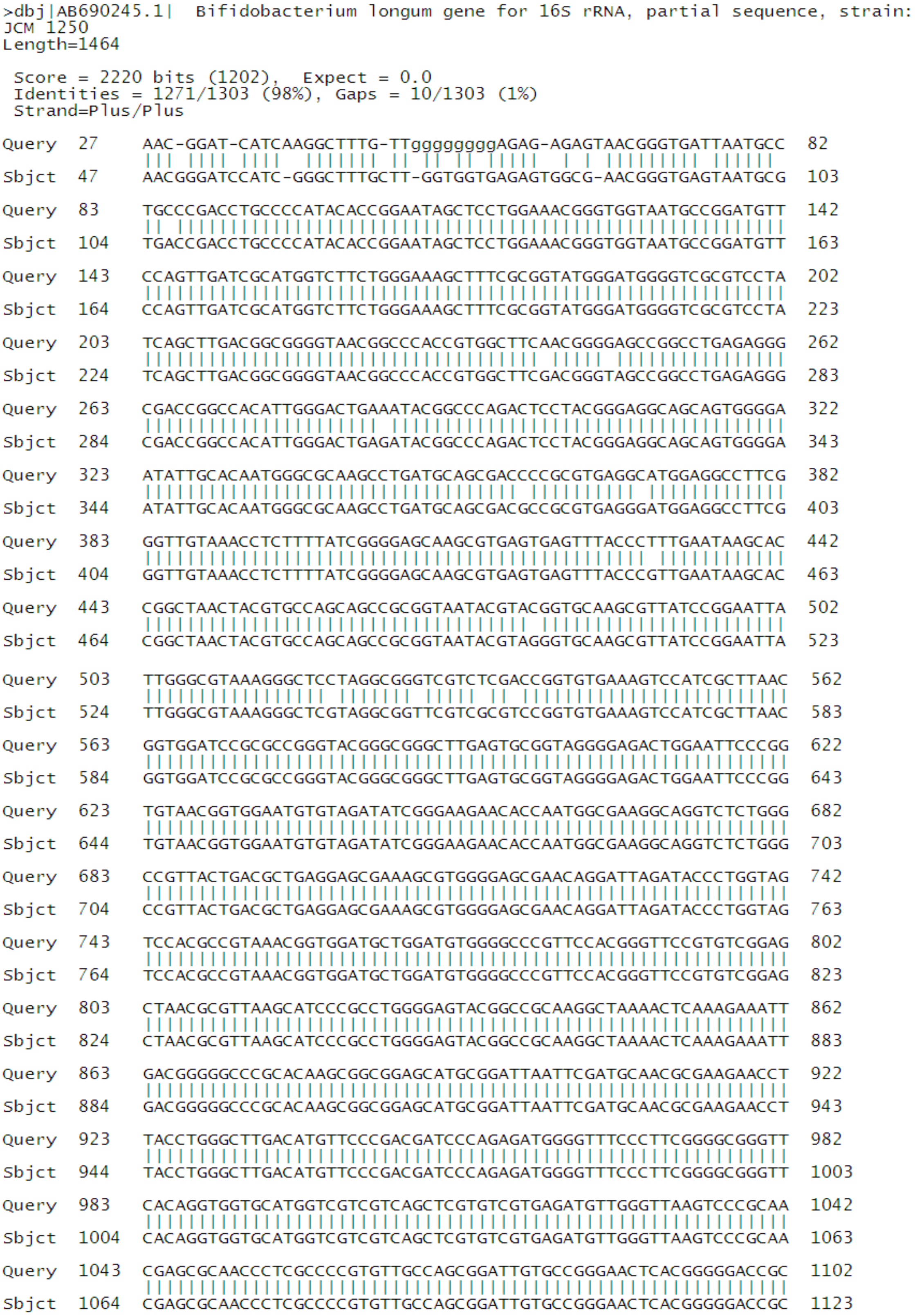
2. The similarly alignment 16S rRNA gene sequence of *Bifidobacterium* spp. isolated from breast milk with the 16S rRNA gene sequence of *Bifidobacterium* spp. published in NCBI data bank.



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>dbj|AB690245.1|  Bifidobacterium longum gene for 16S rRNA, partial sequence, strain:
JCM 1250
Length=1464

 Score = 2220 bits (1202),  Expect = 0.0
 Identities = 1271/1303 (98%), Gaps = 10/1303 (1%)
 Strand=Plus/Plus

Query  27    AAC-GGAT-CATCAAGGCTTTG-TTggggggggAGAG-AGAGTAACGGGTGATTAATGCC  82
             ||| |||| ||||  ||||||| || || || |||||  | | ||||||||| ||||||
Sbjct  47    AACGGGATCCATC-GGGCTTTGCTT-GGTGGTGAGAGTGGCG-AACGGGTGAGTAATGCG  103

Query  83    TGCCCGACCTGCCCCATACACCGGAATAGCTCCTGGAAACGGGTGGTAATGCCGGATGTT  142
             || |||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  104   TGACCGACCTGCCCCATACACCGGAATAGCTCCTGGAAACGGGTGGTAATGCCGGATGTT  163

Query  143   CCAGTTGATCGCATGGTCTTCTGGGAAAGCTTTCGCGGTATGGGATGGGGTCGCGTCCTA  202
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  164   CCAGTTGATCGCATGGTCTTCTGGGAAAGCTTTCGCGGTATGGGATGGGGTCGCGTCCTA  223

Query  203   TCAGCTTGACGGCGGGGTAACGGCCCACCGTGGCTTCAACGGGGAGCCGGCCTGAGAGGG  262
             ||||||||||||||||||||||||||||||||||||| ||||| ||||||||||||||||
Sbjct  224   TCAGCTTGACGGCGGGGTAACGGCCCACCGTGGCTTCGACGGGTAGCCGGCCTGAGAGGG  283

Query  263   CGACCGGCCACATTGGGACTGAAATACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGA  322
             |||||||||||||||||||||| |||||||||||||||||||||||||||||||||||||
Sbjct  284   CGACCGGCCACATTGGGACTGAGATACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGA  343

Query  323   ATATTGCACAATGGGCGCAAGCCTGATGCAGCGACCCCGCGTGAGGCATGGAGGCCTTCG  382
             ||||||||||||||||||||||||||||||||||| |||||||||| |||||||||||||
Sbjct  344   ATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGAGGGATGGAGGCCTTCG  403

Query  383   GGTTGTAAACCTCTTTTATCGGGGAGCAAGCGTGAGTGAGTTTACCCTTTGAATAAGCAC  442
             ||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  404   GGTTGTAAACCTCTTTTATCGGGGAGCAAGCGTGAGTGAGTTTACCCGTTGAATAAGCAC  463

Query  443   CGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTACGGTGCAAGCGTTATCCGGAATTA  502
             |||||||||||||||||||||||||||||||||||| |||||||||||||||||||||||
Sbjct  464   CGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTATCCGGAATTA  523

Query  503   TTGGGCGTAAAGGGCTCCTAGGCGGGTCGTCTCGACCGGTGTGAAAGTCCATCGCTTAAC  562
             ||||||||||||||||| ||||||| ||||| || |||||||||||||||||||||||||
Sbjct  524   TTGGGCGTAAAGGGCTCGTAGGCGGTTCGTCGCGTCCGGTGTGAAAGTCCATCGCTTAAC  583

Query  563   GGTGGATCCGCGCCGGGTACGGGCGGGCTTGAGTGCGGTAGGGGAGACTGGAATTCCCGG  622
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  584   GGTGGATCCGCGCCGGGTACGGGCGGGCTTGAGTGCGGTAGGGGAGACTGGAATTCCCGG  643

Query  623   TGTAACGGTGGAATGTGTAGATATCGGGAAGAACACCAATGGCGAAGGCAGGTCTCTGGG  682
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  644   TGTAACGGTGGAATGTGTAGATATCGGGAAGAACACCAATGGCGAAGGCAGGTCTCTGGG  703

Query  683   CCGTTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAG  742
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  704   CCGTTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAG  763

Query  743   TCCACGCCGTAAACGGTGGATGCTGGATGTGGGGCCCGTTCCACGGGTTCCGTGTCGGAG  802
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  764   TCCACGCCGTAAACGGTGGATGCTGGATGTGGGGCCCGTTCCACGGGTTCCGTGTCGGAG  823

Query  803   CTAACGCGTTAAGCATCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGAAATT  862
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  824   CTAACGCGTTAAGCATCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGAAATT  883

Query  863   GACGGGGGCCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCT  922
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  884   GACGGGGGCCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCT  943

Query  923   TACCTGGGCTTGACATGTTCCCGACGATCCCAGAGATGGGGTTTCCCTTCGGGGCGGGTT  982
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  944   TACCTGGGCTTGACATGTTCCCGACGATCCCAGAGATGGGGTTTCCCTTCGGGGCGGGTT  1003

Query  983   CACAGGTGGTGCATGGTCGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAA  1042
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1004  CACAGGTGGTGCATGGTCGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAA  1063

Query  1043  CGAGCGCAACCCTCGCCCCGTGTTGCCAGCGGATTGTGCCGGGAACTCACGGGGGACCGC  1102
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1064  CGAGCGCAACCCTCGCCCCGTGTTGCCAGCGGATTGTGCCGGGAACTCACGGGGGACCGC  1123
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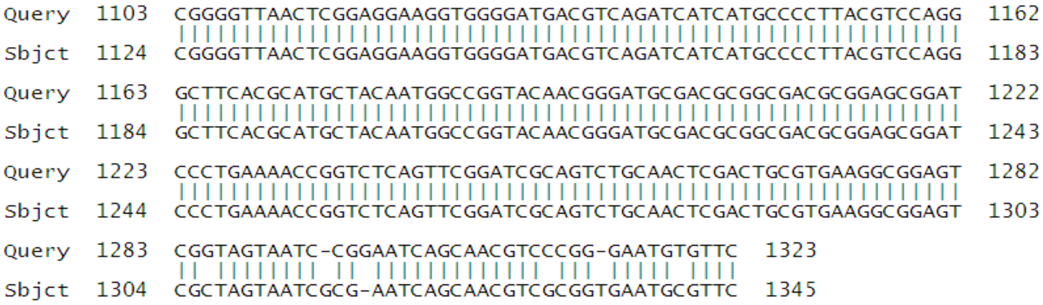

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Query  1103  CGGGGTTAACTCGGAGGAAGGTGGGGATGACGTCAGATCATCATGCCCCTTACGTCCAGG  1162
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1124  CGGGGTTAACTCGGAGGAAGGTGGGGATGACGTCAGATCATCATGCCCCTTACGTCCAGG  1183

Query  1163  GCTTCACGCATGCTACAATGGCCGGTACAACGGGATGCGACGCGGCGACGCGGAGCGGAT  1222
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1184  GCTTCACGCATGCTACAATGGCCGGTACAACGGGATGCGACGCGGCGACGCGGAGCGGAT  1243

Query  1223  CCCTGAAAACCGGTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGGCGGAGT  1282
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1244  CCCTGAAAACCGGTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGGCGGAGT  1303

Query  1283  CGGTAGTAATC-CGGAATCAGCAACGTCCCGG-GAATGTGTTC  1323
             || |||||||| || ||||||||||||| ||| ||||| ||||
Sbjct  1304  CGCTAGTAATCGCG-AATCAGCAACGTCGCGGTGAATGCGTTC  1345
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>gb|GU936674.1|  Bifidobacterium bifidum strain R0071 16S ribosomal RNA gene,
partial sequence
Length=1368

 Score = 2224 bits (1204),  Expect = 0.0
 Identities = 1274/1305 (98%), Gaps = 15/1305 (1%)
 Strand=Plus/Plus

Query  29    GGACTCGATCGCGGCTTTGCCTGGTGGTGAGAGTGGCGAACGGGTGAGTAATGCGTGACC  88
             ||| || |||| |||||||| |||||||||||||||||||||||||||||||||||||||
Sbjct  4     GGA-TCCATCG-GGCTTTGCTTGGTGGTGAGAGTGGCGAACGGGTGAGTAATGCGTGACC  61

Query  89    GACCTGCCCCATGCTCCGGAATAGCTCCTGGAAACGGGTGGTAATGCCGGATGTTCCACA  148
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  62    GACCTGCCCCATGCTCCGGAATAGCTCCTGGAAACGGGTGGTAATGCCGGATGTTCCACA  121

Query  149   TGATCGCATGTGATTGTGGGAAAGATTCTATCGGCGTGGATGGGGTCGCGTCCTATCAG  208
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  122   TGATCGCATGTGATTGTGGGAAAGATTCTATCGGCGTGGATGGGGTCGCGTCCTATCAG  181

Query  209   CTTGTTGGTGAGGTAACGGCTCACCAAGGCTTCGACGGGTAGCCGGCCTGAGAGGGCGAC  268
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  182   CTTGTTGGTGAGGTAACGGCTCACCAAGGCTTCGACGGGTAGCCGGCCTGAGAGGGCGAC  241

Query  269   CGGCCACATTGGGACTGAGATACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATAT  328
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Sbjct  242   CGGCCACATTGGGACTGAGATACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATAT  301

Query  329   TGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGAGGGATGGAGGCCTTCGGGTT  388
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Sbjct  302   TGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGAGGGATGGAGGCCTTCGGGTT  361

Query  389   GTAAACCTCTTTTGTTTGGGAGCAAGCCTTCGGGTGAGTGTACCTTTCGAATAAGCGCCG  448
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Sbjct  362   GTAAACCTCTTTTGTTTGGGAGCAAGCCTTCGGGTGAGTGTACCTTTCGAATAAGCGCCG  421

Query  449   GCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTATCCGGATTTATT  508
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Sbjct  422   GCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTATCCGGATTTATT  481

Query  509   GGGCGTAAAGGGCTCGTAGGCGGCTCGTCGCGTCCGGTGTGAAAGTCCATCGCTTAACGG  568
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Sbjct  482   GGGCGTAAAGGGCTCGTAGGCGGCTCGTCGCGTCCGGTGTGAAAGTCCATCGCTTAACGG  541

Query  569   TGGATCTGCGCCGGGTACGGGCGGGCTGGAGTGCGGTAGGGGAGACTGGAATTCCCGGTG  628
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Sbjct  542   TGGATCTGCGCCGGGTACGGGCGGGCTGGAGTGCGGTAGGGGAGACTGGAATTCCCGGTG  601

Query  629   TAACGGTGGAATGTGTAGATATCGGGAAGAACACCGATGGCGAAGGCAGGTCTCTGGGCC  688
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Sbjct  602   TAACGGTGGAATGTGTAGATATCGGGAAGAACACCGATGGCGAAGGCAGGTCTCTGGGCC  661

Query  689   GTCACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTC  748
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Sbjct  662   GTCACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTC  721

Query  749   CACGCCGTAAACGGTGGACGCTGGATGTGGGGCACGTTCCACGTGTTCCGTGTCTGAACT  808
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Sbjct  722   CACGCCGTAAACGGTGGACGCTGGATGTGGGGCACGTTCCACGTGTTCCGTGTCGGAGCT  781

Query  809   AACGCGTTAAGCGTCCCGCCTGGGCAGTACGGCCCGCAAGGCTAAAACTCAAAGAAATTG  868
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Sbjct  782   AACGCGTTAAGCGTCCCGCCTGGGGAGTACGGCC-GCAAGGCTAAAACTCAAAGAAATTG  840

Query  869   ACCGGGGGCCCTACACAAAGCGGGGGGAGCATGCAGAATTAACTTCGATTTCAACCCGAA  928
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Sbjct  841   AC-GGGGGCCCG-CACAA-GCGGCGG-AGCATGCGGA-TTAA-TTCGATG-CAACGCGAA  893

Query  929   AAGACCCTTACCTGGGCTTGACATGTTCCCGACGACGCCAGAGATGGCGTTTCCCTTCGG  988
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Sbjct  894   GA-ACC-TTACCTGGGCTTGACATGTTCCCGACGACGCCAGAGATGGCGTTTCCCTTCGG  951

Query  989   GGCGGGTTCACAGGTGGTTCATGGTCGTCGTCAGCTCGTGTTGTGAGATGTTGGGTTAAG  1048
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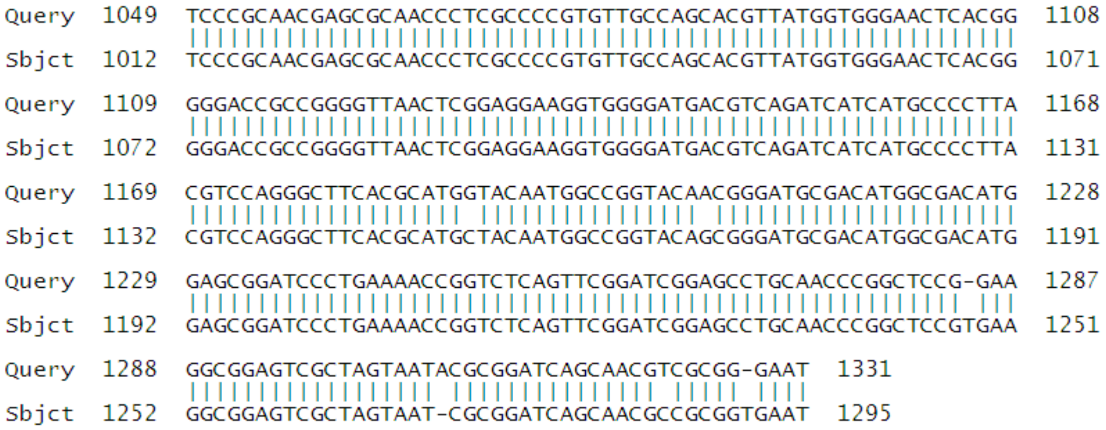

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Query  1049  TCCCGCAACGAGCGCAACCCTCGCCCCGTGTTGCCAGCACGTTATGGTGGGAACTCACGG  1108
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Sbjct  1012  TCCCGCAACGAGCGCAACCCTCGCCCCGTGTTGCCAGCACGTTATGGTGGGAACTCACGG  1071

Query  1109  GGGACCGCCGGGGTTAACTCGGAGGAAGGTGGGGATGACGTCAGATCATCATGCCCCTTA  1168
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Sbjct  1072  GGGACCGCCGGGGTTAACTCGGAGGAAGGTGGGGATGACGTCAGATCATCATGCCCCTTA  1131

Query  1169  CGTCCAGGGCTTCACGCATGGTACAATGGCCGGTACAACGGGATGCGACATGGCGACATG  1228
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Sbjct  1132  CGTCCAGGGCTTCACGCATGCTACAATGGCCGGTACAGCGGGATGCGACATGGCGACATG  1191

Query  1229  GAGCGGATCCCTGAAAACCGGTCTCAGTTCGGATCGGAGCCTGCAACCCGGCTCCG-GAA  1287
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||
Sbjct  1192  GAGCGGATCCCTGAAAACCGGTCTCAGTTCGGATCGGAGCCTGCAACCCGGCTCCGTGAA  1251

Query  1288  GGCGGAGTCGCTAGTAATACGCGGATCAGCAACGTCGCGG-GAAT  1331
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Sbjct  1252  GGCGGAGTCGCTAGTAAT-CGCGGATCAGCAACGCCGCGGTGAAT  1295
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>gb|HQ259740.1|  Bifidobacterium breve strain LCR5 16S ribosomal RNA gene, partial
sequence
Length=1393

 Score = 2314 bits (1253),  Expect = 0.0
 Identities = 1282/1296 (99%), Gaps = 1/1296 (0%)
 Strand=Plus/Plus

Query  32    CCATCGAGCTTTGCTTGGTGGTGAGAGTGGCGAACGGGTGAGTAATGCGTGACCGACCTG  91
             |||||| ||||||| |||||||||||||||||||||||||||||||||||||||||||||
Sbjct  46    CCATCGGGCTTTGCCTGGTGGTGAGAGTGGCGAACGGGTGAGTAATGCGTGACCGACCTG  105

Query  92    CCCCATGCACCGGAATAGCTCCTGGAAACGGGTGGTAATGCCGGATGCTCCATCACACCG  151
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  106   CCCCATGCACCGGAATAGCTCCTGGAAACGGGTGGTAATGCCGGATGCTCCATCACACCG  165

Query  152   CATGGTGTGTTGGGAAAGCCTTTGCGGCATGGGATGGGGTCGCGTCCTATCAGCTTGATG  211
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Sbjct  166   CATGGTGTGTTGGGAAAGCCTTTGCGGCATGGGATGGGGTCGCGTCCTATCAGCTTGATG  225

Query  212   GCGGGGTAACGGCCCACCATGGCTTCGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCAC  271
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Sbjct  226   GCGGGGTAACGGCCCACCATGGCTTCGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCAC  285

Query  272   ATTGGGACTGAGATACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAA  331
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Sbjct  286   ATTGGGACTGAGATACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAA  345

Query  332   TGGGCGCAAGCCTGATGCAGCGACGCCGCGTGAGGGATGGAGGCCTTCGGGTTGTAAACC  391
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Sbjct  346   TGGGCGCAAGCCTGATGCAGCGACGCCGCGTGAGGGATGGAGGCCTTCGGGTTGTAAACC  405

Query  392   TCTTTTGTTAGGGAGCAAGGCATTTTGTGTTGAGTGTACCTTTCGAATAAGCACCGGCTA  451
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Sbjct  406   TCTTTTGTTAGGGAGCAAGGCACTTTGTGTTGAGTGTACCTTTCGAATAAGCACCGGCTA  465

Query  452   ACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTATCCGGAATTATTGGGC  511
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Sbjct  466   ACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTATCCGGAATTATTGGGC  525

Query  512   GTAAAGGGCTCGTAAGCGGTTCGTCCCGTCCGGTGTGAAAGTCCATCGCTTAACGGTGGA  571
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Sbjct  526   GTAAAGGGCTCGTAGGCGGTTCGTCGCGTCCGGTGTGAAAGTCCATCGCTTAACGGTGGA  585

Query  572   TCCGCGCCGGGTACGGGCGGGCTTGAGTGCGGTAGGGGAGACTGGAATTCCCGGTGTAAC  631
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Sbjct  586   TCCGCGCCGGGTACGGGCGGGCTTGAGTGCGGTAGGGGAGACTGGAATTCCCGGTGTAAC  645

Query  632   GGTGGAATGTGTAGATATCGGGAAGAACACCAATGGCGAAGGCAGGTCTCTGGGCCGTTA  691
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  646   GGTGGAATGTGTAGATATCGGGAAGAACACCAATGGCGAAGGCAGGTCTCTGGGCCGTTA  705

Query  692   CTGACGCTGAAGAACGAAAGCCTGGGGAGCCAACAGGATTAGATACCCTGGTAGTCCACG  751
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Sbjct  706   CTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACG  765

Query  752   CCGTAAACGGTGGATGCTGGATGTGGGGCCCGTTCCACGGGTTCCGTGTCCGAACTAACG  811
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Sbjct  766   CCGTAAACGGTGGATGCTGGATGTGGGGCCCGTTCCACGGGTTCCGTGTCGGAGCTAACG  825

Query  812   CGTTAAGCATCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGAAATTGACGGG  871
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  826   CGTTAAGCATCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGAAATTGACGGG  885

Query  872   GGCCCGCACAAGCGGCGGAACATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCTG  931
             ||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  886   GGCCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCTG  945

Query  932   GGCTTGACATGTTCCCGACGATCCCAGAGATGGGGTTTCCCTTCGGGGCGGGTTCACAGG  991
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  946   GGCTTGACATGTTCCCGACGATCCCAGAGATGGGGTTTCCCTTCGGGGCGGGTTCACAGG  1005

Query  992   TGGTGCATGGTCGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCG  1051
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Sbjct  1006  TGGTGCATGGTCGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCG  1065

Query  1052  CAACCCTCGCCCCGTGTTGCCAGCGGATTGTGCCGGGAACTCACGGGGGACCGCCGGGGT  1111
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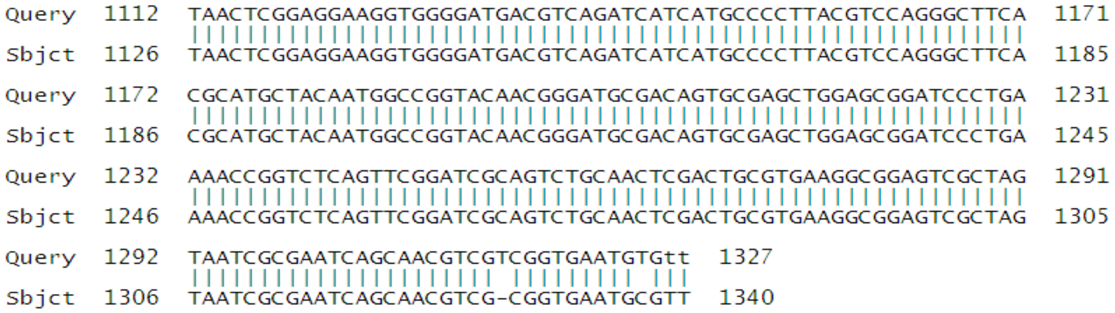

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Query  1112  TAACTCGGAGGAAGGTGGGGATGACGTCAGATCATCATGCCCCTTACGTCCAGGGCTTCA  1171
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Sbjct  1126  TAACTCGGAGGAAGGTGGGGATGACGTCAGATCATCATGCCCCTTACGTCCAGGGCTTCA  1185

Query  1172  CGCATGCTACAATGGCCGGTACAACGGGATGCGACAGTGCGAGCTGGAGCGGATCCCTGA  1231
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Sbjct  1186  CGCATGCTACAATGGCCGGTACAACGGGATGCGACAGTGCGAGCTGGAGCGGATCCCTGA  1245

Query  1232  AAACCGGTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGGCGGAGTCGCTAG  1291
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Sbjct  1246  AAACCGGTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGGCGGAGTCGCTAG  1305

Query  1292  TAATCGCGAATCAGCAACGTCGTCGGTGAATGTGtt  1327
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>ref|NR_037117.1|  Bifidobacterium pseudocatenulatum strain B1279 16S ribosomal
RNA, partial sequence
Length=1513

 Score = 2361 bits (1278),  Expect = 0.0
 Identities = 1294/1302 (99%), Gaps = 0/1302 (0%)
 Strand=Plus/Plus

Query  24    AACGGAATCCATCAGGCTTTGCTTGGTGGTGAGGGTGGCGAACGGGTGAGTAATGCGTGA  83
             ||||| ||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct  56    AACGGGATCCATCAGGCTTTGCTTGGTGGTGAGAGTGGCGAACGGGTGAGTAATGCGTGA  115

Query  84    CCGACCTGCCCCATACACCGGAATAGCTCCTGGAAACGGGTGGTAATGCCGGATGCTCCG  143
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  116   CCGACCTGCCCCATACACCGGAATAGCTCCTGGAAACGGGTGGTAATGCCGGATGCTCCG  175

Query  144   ACTCCTCGCATGGGGTGTCGGGAAAGATTTCATCGGTATGGATGGGGTCGCGTCCTATC  203
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  176   ACTCCTCGCATGGGGTGTCGGGAAAGATTTCATCGGTATGGATGGGGTCGCGTCCTATC  235

Query  204   AGGTAGTCGGCGGGGTAACGGCCCACCGAGCCTACGACGGGTAGCCGGCCTGAGAGGGCG  263
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Sbjct  236   AGGTAGTCGGCGGGGTAACGGCCCACCGAGCCTACGACGGGTAGCCGGCCTGAGAGGGCG  295

Query  264   ACCGGCCACATTGGGACTGAGATACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAAT  323
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Sbjct  296   ACCGGCCACATTGGGACTGAGATACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAAT  355

Query  324   ATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGCGGGATGACGGCCTTCGGG  383
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Sbjct  356   ATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGCGGGATGACGGCCTTCGGG  415

Query  384   TTGTAAACCGCTTTTGATCGGGAGCAAGCCTTCGGGTGAGTGTACCTTTCGAATAAGCAC  443
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Sbjct  416   TTGTAAACCGCTTTTGATCGGGAGCAAGCCTTCGGGTGAGTGTACCTTTCGAATAAGCAC  475

Query  444   CGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTATCCGGAATTA  503
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Sbjct  476   CGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTATCCGGAATTA  535

Query  504   TTGGGCGTAAAGGGCTCGTAGGCGGTTCGTCGCGTCCGGTGTGAAAGTCCATCGCTTAAC  563
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Sbjct  536   TTGGGCGTAAAGGGCTCGTAGGCGGTTCGTCGCGTCCGGTGTGAAAGTCCATCGCTTAAC  595

Query  564   GGTGGATCTGCGCCGGGTACGGGCGGGCTGGAGTGCGGTAGGGGAGACTGGAATTCCCGG  623
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Sbjct  596   GGTGGATCTGCGCCGGGTACGGGCGGGCTGGAGTGCGGTAGGGGAGACTGGAATTCCCGG  655

Query  624   TGTAACGGTGGAATGTGTAGATATCGGGAAGAACACCAATGGCGAAGGCAGGTCTCTGGG  683
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Sbjct  656   TGTAACGGTGGAATGTGTAGATATCGGGAAGAACACCAATGGCGAAGGCAGGTCTCTGGG  715

Query  684   CCGTTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAG  743
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Sbjct  716   CCGTTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAG  775

Query  744   TCCACGCCGTAAACGGTGGATGCTGGATGTGGGGCCCGTTCCACGGGTTCCGTGTCGGAG  803
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Sbjct  776   TCCACGCCGTAAACGGTGGATGCTGGATGTGGGGCCCGTTCCACGGGTTCCGTGTCGGAG  835

Query  804   CTAACGCGTTAAGCATCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGAAATT  863
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Sbjct  836   CTAACGCGTTAAGCATCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGAAATT  895

Query  864   GACGGGGGCCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCT  923
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Sbjct  896   GACGGGGGCCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCT  955

Query  924   TACCTGGGCTTGACATGTTCCCGACAGCGGCAGAGATGTCGCTTCCCTTCGGGGCGGGTT  983
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Sbjct  956   TACCTGGGCTTGACATGTTCCCGACAGCCGTAGAGATATGGCCTCCCTTCGGGGCGGGTT  1015

Query  984   CACAGGTGGTGCATGGTCGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAA  1043
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Sbjct  1016  CACAGGTGGTGCATGGTCGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAA  1075

Query  1044  CGAGCGCAACCCTCGCCCTGTGTTGCCAGCACGTCGTGGTGGGAACTCACGGGGGACCGC  1103
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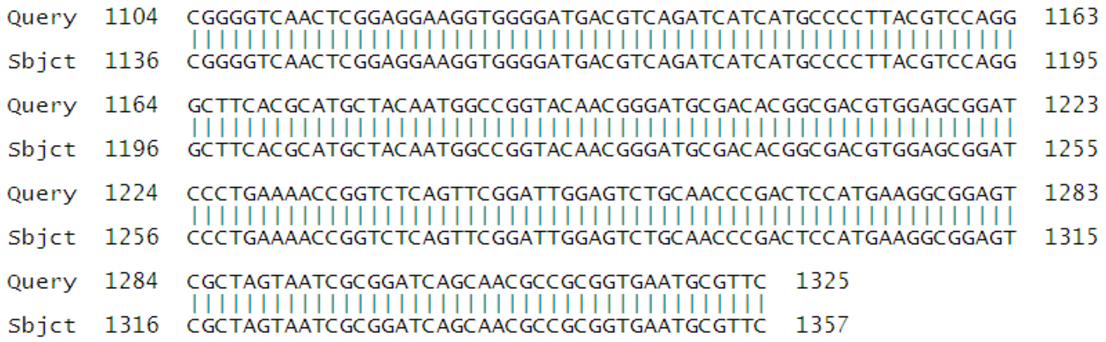

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Query  1104  CGGGGTCAACTCGGAGGAAGGTGGGGATGACGTCAGATCATCATGCCCCTTACGTCCAGG  1163
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Sbjct  1136  CGGGGTCAACTCGGAGGAAGGTGGGGATGACGTCAGATCATCATGCCCCTTACGTCCAGG  1195

Query  1164  GCTTCACGCATGCTACAATGGCCGGTACAACGGGATGCGACACGGCGACGTGGAGCGGAT  1223
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Sbjct  1196  GCTTCACGCATGCTACAATGGCCGGTACAACGGGATGCGACACGGCGACGTGGAGCGGAT  1255

Query  1224  CCCTGAAAACCGGTCTCAGTTCGGATTGGAGTCTGCAACCCGACTCCATGAAGGCGGAGT  1283
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Sbjct  1256  CCCTGAAAACCGGTCTCAGTTCGGATTGGAGTCTGCAACCCGACTCCATGAAGGCGGAGT  1315

Query  1284  CGCTAGTAATCGCGGATCAGCAACGCCGCGGTGAATGCGTTC  1325
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Sbjct  1316  CGCTAGTAATCGCGGATCAGCAACGCCGCGGTGAATGCGTTC  1357
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>gb|GU361819.1|  Bifidobacterium dentium strain KCTC 3222 16S ribosomal RNA gene,
partial sequence
Length=1483

 Score = 2314 bits (1253),  Expect = 0.0
 Identities = 1274/1284 (99%), Gaps = 1/1284 (0%)
 Strand=Plus/Plus

Query  33    GGGGGTTCGCTTCCGGGTGAGAGTGGCGAACGGGTGAGTAATGCGTGACCGACCTGCCCC  92
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Sbjct  49    GGGGGTTCGCCTCCGGGTGAGAGTGGCGAACGGGTGAGTAATGCGTGACCGACCTGCCCC  108

Query  93    ATACACCGGAATAGCTCCTGGAAACGGGTGGTAATGCCGGATGCTCCGGTTGGATGCATG  152
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Sbjct  109   ATACACCGGAATAGCTCCTGGAAACGGGTGGTAATGCCGGATGCTCCGGTTGGATGCATG  168

Query  153   TCCTTCCGGGAAA-GATCCGTCGGTATGGGATGGGGTCGCGTCCTATCAGCTTGATGGCG  211
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Sbjct  169   TCCTTCCGGGAAAGGTTCCATCGGTATGGGATGGGGTCGCGTCCTATCAGCTTGATGGCG  228

Query  212   GGGTAACGGCCCACCATGGCTTCGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACATT  271
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Sbjct  229   GGGTAACGGCCCACCATGGCTTCGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACATT  288

Query  272   GGGACTGAGATACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGG  331
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Sbjct  289   GGGACTGAGATACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGG  348

Query  332   GCGCAAGCCTGATGCAGCGACGCCGCGTGCGGGATGGAGGCCTTCGGGTTGTAAACCGCT  391
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Sbjct  349   GCGCAAGCCTGATGCAGCGACGCCGCGTGCGGGATGGAGGCCTTCGGGTTGTAAACCGCT  408

Query  392   TTTGATCGGGAGCAAGCCCTTCGGGGTGAGTGTACCTTTCGAATAAGCACCGGCTAACTA  451
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Sbjct  409   TTTGATCGGGAGCAAGCCCTTCGGGGTGAGTGTACCCTTCGAATAAGCACCGGCTAACTA  468

Query  452   CGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTATCCGGAATTATTGGGCGTAA  511
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Sbjct  469   CGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTATCCGGAATTATTGGGCGTAA  528

Query  512   AGGGCTCGTAGGCGGTTCGTCGCGTCCGGTGTGAAAGCCCATCGCTTAACGGTGGGTCTG  571
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  529   AGGGCTCGTAGGCGGTTCGTCGCGTCCGGTGTGAAAGCCCATCGCTTAACGGTGGGTCTG  588

Query  572   CGCCGGGTACGGGCGGGCTGGAGTGCGGTAGGGGAGACTGGAATTCCCGGTGTAACGGTG  631
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  589   CGCCGGGTACGGGCGGGCTGGAGTGCGGTAGGGGAGACTGGAATTCCCGGTGTAACGGTG  648

Query  632   GAATGTGTAGATATCGGGAAGAACACCAATGGCGAAGGCAGGTCTCTGGGCCGTCACTGA  691
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  649   GAATGTGTAGATATCGGGAAGAACACCAATGGCGAAGGCAGGTCTCTGGGCCGTCACTGA  708

Query  692   CGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGT  751
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Sbjct  709   CGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGT  768

Query  752   AAACGGTGGATGCTGGATGTGGGGCCCGTTCCACGGGTTCCGTGTCGGAGCTAACGCGTT  811
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  769   AAACGGTGGATGCTGGATGTGGGGCCCGTTCCACGGGTTCCGTGTCGGAGCTAACGCGTT  828

Query  812   AAGCATCCCGCCTGGAGAGTACGGCCGCAAGGCTAACACTCAAAGAAATTGACGGGGGCC  871
             ||||||||||||||| |||||||||||||||||||| |||||||||||||||||||||||
Sbjct  829   AAGCATCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGAAATTGACGGGGGCC  888

Query  872   CGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAAAACCTTACCTGGGCT  931
             |||||||||||||||||||||||||||||||||||||||||||| |||||||||||||||
Sbjct  889   CGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCTGGGCT  948

Query  932   TGACATGTTCCCGACAGCCGTAGAGATATGGCCTCCCTTCGGGGCGGGTTCACAGGTGGT  991
             ||||||||||||||| |||||||||||| |||||||||||||||||||||||||||||||
Sbjct  949   TGACATGTTCCCGACGGCCGTAGAGATACGGCCTCCCTTCGGGGCGGGTTCACAGGTGGT  1008

Query  992   GCATGGTCGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAAC  1051
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1009  GCATGGTCGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAAC  1068
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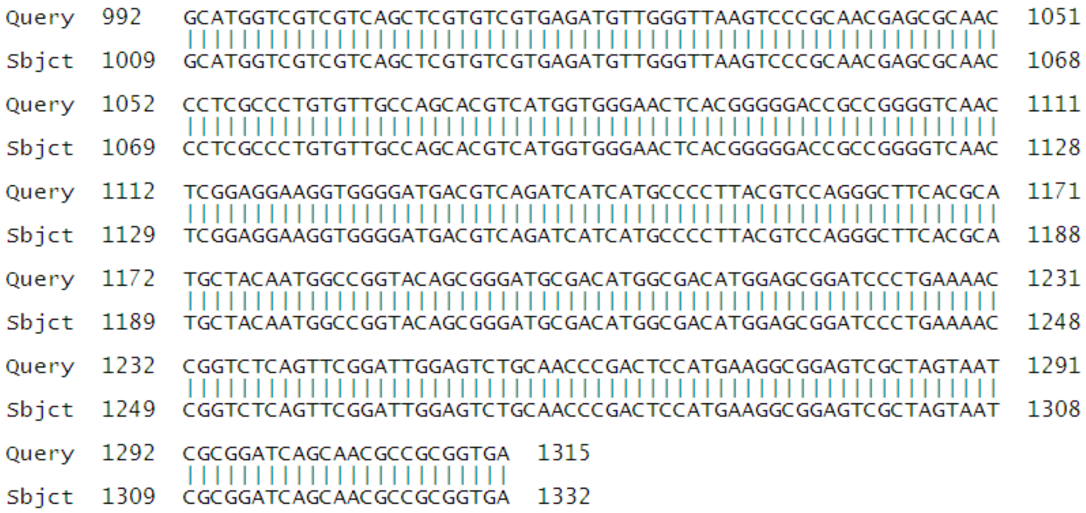

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Query  992   GCATGGTCGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAAC  1051
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Sbjct  1009  GCATGGTCGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAAC  1068

Query  1052  CCTCGCCCTGTGTTGCCAGCACGTCATGGTGGGAACTCACGGGGGACCGCCGGGGTCAAC  1111
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1069  CCTCGCCCTGTGTTGCCAGCACGTCATGGTGGGAACTCACGGGGGACCGCCGGGGTCAAC  1128

Query  1112  TCGGAGGAAGGTGGGGATGACGTCAGATCATCATGCCCCTTACGTCCAGGGCTTCACGCA  1171
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1129  TCGGAGGAAGGTGGGGATGACGTCAGATCATCATGCCCCTTACGTCCAGGGCTTCACGCA  1188

Query  1172  TGCTACAATGGCCGGTACAGCGGGATGCGACATGGCGACATGGAGCGGATCCCTGAAAAC  1231
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1189  TGCTACAATGGCCGGTACAGCGGGATGCGACATGGCGACATGGAGCGGATCCCTGAAAAC  1248

Query  1232  CGGTCTCAGTTCGGATTGGAGTCTGCAACCCGACTCCATGAAGGCGGAGTCGCTAGTAAT  1291
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1249  CGGTCTCAGTTCGGATTGGAGTCTGCAACCCGACTCCATGAAGGCGGAGTCGCTAGTAAT  1308

Query  1292  CGCGGATCAGCAACGCCGCGGTGA  1315
             ||||||||||||||||||||||||
Sbjct  1309  CGCGGATCAGCAACGCCGCGGTGA  1332
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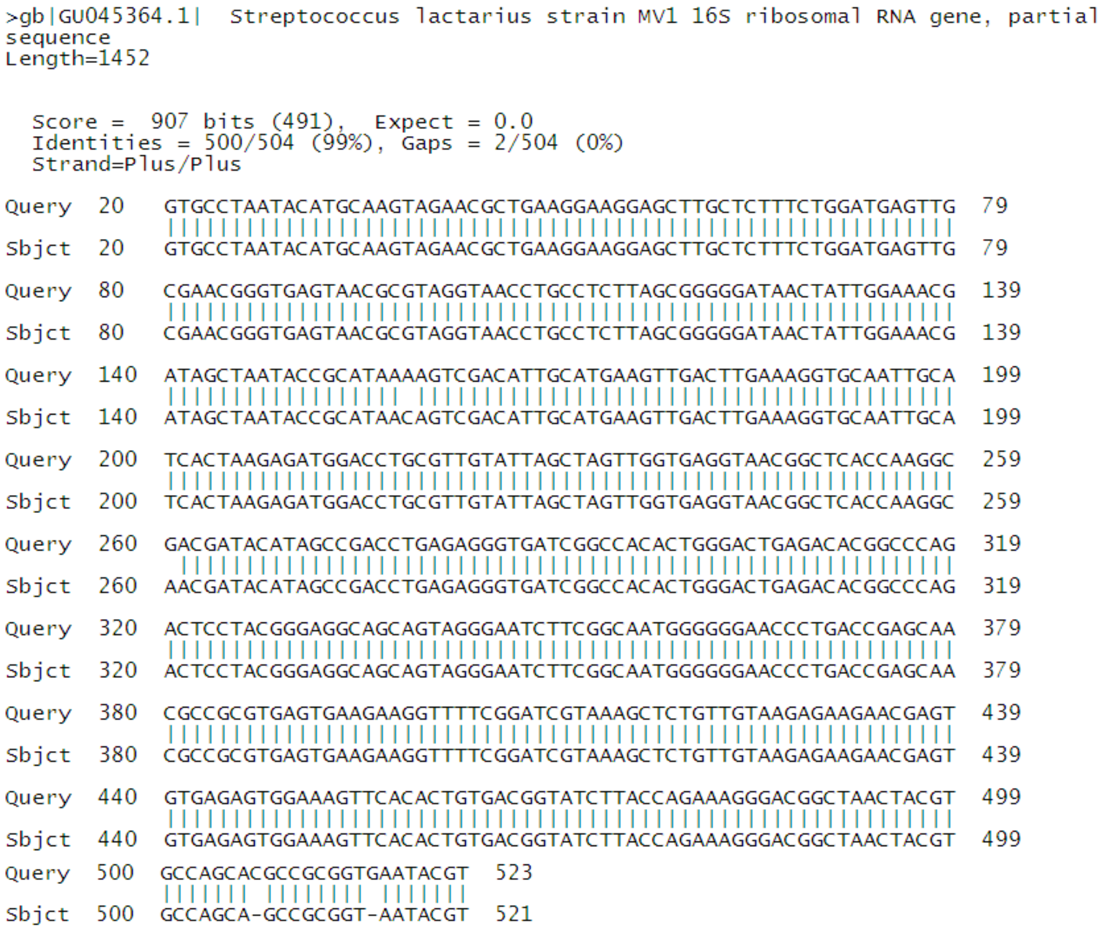
3. The similarly alignment 16S rRNA gene sequence of *Streptococcus* spp. isolated from breast milk with the 16S rRNA gene sequence of *Streptococcus* spp. published in NCBI data bank.



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>gb|GU045364.1|  Streptococcus lactarius strain MV1 16S ribosomal RNA gene, partial
sequence
Length=1452

 Score =  907 bits (491),  Expect = 0.0
 Identities = 500/504 (99%), Gaps = 2/504 (0%)
 Strand=Plus/Plus

Query  20   GTGCCTAATACATGCAAGTAGAACGCTGAAGGAAGGAGCTTGCTCTTTCTGGATGAGTTG  79
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  20   GTGCCTAATACATGCAAGTAGAACGCTGAAGGAAGGAGCTTGCTCTTTCTGGATGAGTTG  79

Query  80   CGAACGGGTGAGTAACGCGTAGGTAACCTGCCTCTTAGCGGGGGATAACTATTGGAAACG  139
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  80   CGAACGGGTGAGTAACGCGTAGGTAACCTGCCTCTTAGCGGGGGATAACTATTGGAAACG  139

Query  140  ATAGCTAATACCGCATAAAAGTCGACATTGCATGAAGTTGACTTGAAAGGTGCAATTGCA  199
            |||||||||||||||||| |||||||||||||||||||||||||||||||||||||||||
Sbjct  140  ATAGCTAATACCGCATAACAGTCGACATTGCATGAAGTTGACTTGAAAGGTGCAATTGCA  199

Query  200  TCACTAAGAGATGGACCTGCGTTGTATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGC  259
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  200  TCACTAAGAGATGGACCTGCGTTGTATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGC  259

Query  260  GACGATACATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAG  319
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  260  AACGATACATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAG  319

Query  320  ACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGGGGGAACCCTGACCGAGCAA  379
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  320  ACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGGGGGAACCCTGACCGAGCAA  379

Query  380  CGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTAAGAGAAGAACGAGT  439
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  380  CGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTAAGAGAAGAACGAGT  439

Query  440  GTGAGAGTGGAAAGTTCACACTGTGACGGTATCTTACCAGAAAGGGACGGCTAACTACGT  499
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Sbjct  440  GTGAGAGTGGAAAGTTCACACTGTGACGGTATCTTACCAGAAAGGGACGGCTAACTACGT  499
Query  500  GCCAGCACGCCGCGGTGAATACGT  523
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Sbjct  500  GCCAGCA-GCCGCGGT-AATACGT  521
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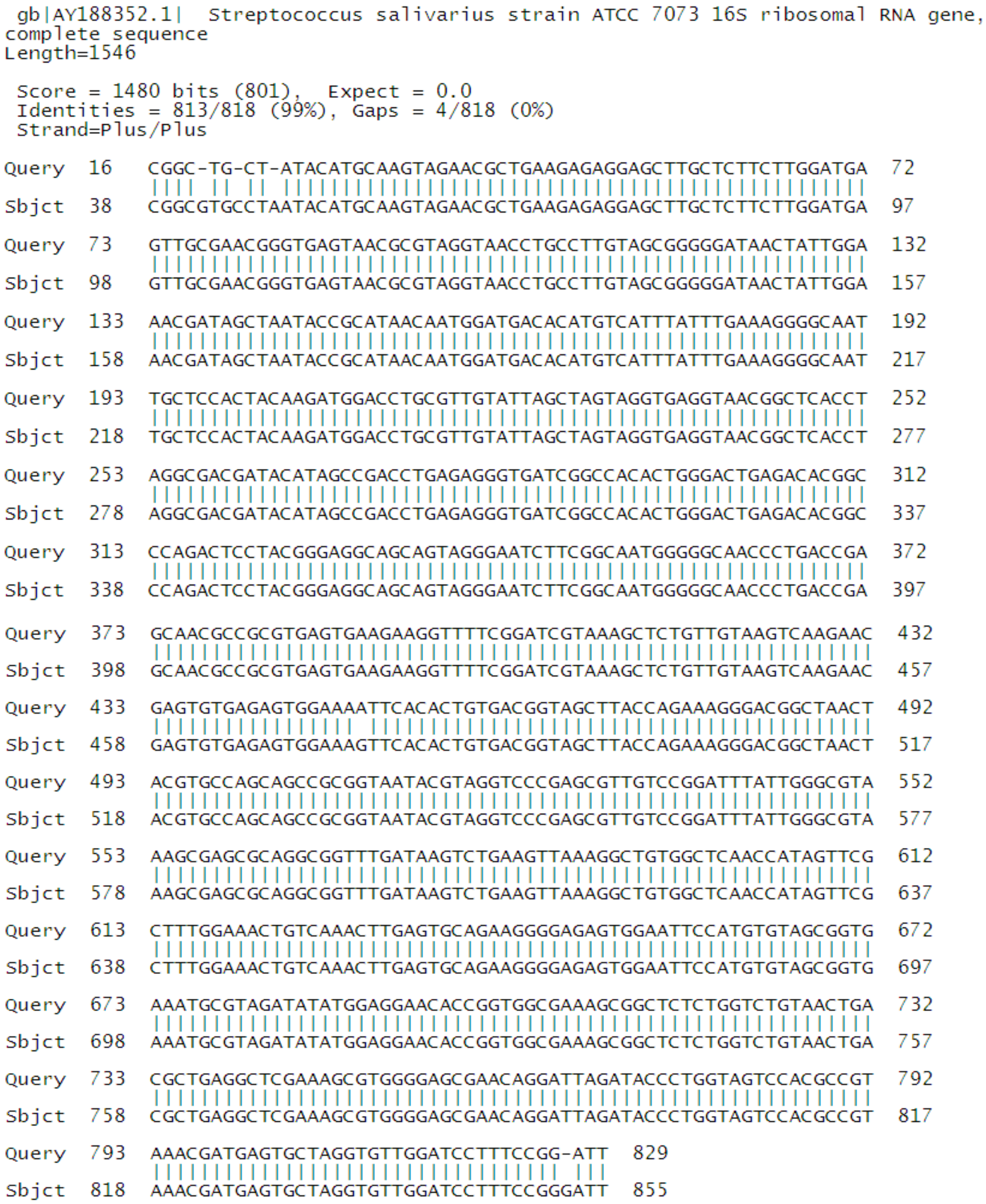

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 gb|AY188352.1|  Streptococcus salivarius strain ATCC 7073 16S ribosomal RNA gene,
complete sequence
Length=1546

 Score = 1480 bits (801),  Expect = 0.0
 Identities = 813/818 (99%), Gaps = 4/818 (0%)
 Strand=Plus/Plus

Query  16   CGGC-TG-CT-ATACATGCAAGTAGAACGCTGAAGAGAGGAGCTTGCTCTTCTTGGATGA  72
            |||| || || |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  38   CGGCGTGCCTAATACATGCAAGTAGAACGCTGAAGAGAGGAGCTTGCTCTTCTTGGATGA  97

Query  73   GTTGCGAACGGGTGAGTAACGCGTAGGTAACCTGCCTTGTAGCGGGGGATAACTATTGGA  132
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  98   GTTGCGAACGGGTGAGTAACGCGTAGGTAACCTGCCTTGTAGCGGGGGATAACTATTGGA  157

Query  133  AACGATAGCTAATACCGCATAACAATGGATGACACATGTCATTTATTTGAAAGGGGCAAT  192
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  158  AACGATAGCTAATACCGCATAACAATGGATGACACATGTCATTTATTTGAAAGGGGCAAT  217

Query  193  TGCTCCACTACAAGATGGACCTGCGTTGTATTAGCTAGTAGGTGAGGTAACGGCTCACCT  252
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  218  TGCTCCACTACAAGATGGACCTGCGTTGTATTAGCTAGTAGGTGAGGTAACGGCTCACCT  277

Query  253  AGGCGACGATACATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGC  312
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  278  AGGCGACGATACATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGC  337

Query  313  CCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGGGGCAACCCTGACCGA  372
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  338  CCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGGGGCAACCCTGACCGA  397

Query  373  GCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTAAGTCAAGAAC  432
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  398  GCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTAAGTCAAGAAC  457

Query  433  GAGTGTGAGAGTGGAAAATTCACACTGTGACGGTAGCTTACCAGAAAGGGACGGCTAACT  492
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Sbjct  458  GAGTGTGAGAGTGGAAAGTTCACACTGTGACGGTAGCTTACCAGAAAGGGACGGCTAACT  517

Query  493  ACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAGCGTTGTCCGGATTTATTGGGCGTA  552
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  518  ACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAGCGTTGTCCGGATTTATTGGGCGTA  577

Query  553  AAGCGAGCGCAGGCGGTTTGATAAGTCTGAAGTTAAAGGCTGTGGCTCAACCATAGTTCG  612
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  578  AAGCGAGCGCAGGCGGTTTGATAAGTCTGAAGTTAAAGGCTGTGGCTCAACCATAGTTCG  637

Query  613  CTTTGGAAACTGTCAAACTTGAGTGCAGAAGGGGAGAGTGGAATTCCATGTGTAGCGGTG  672
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  638  CTTTGGAAACTGTCAAACTTGAGTGCAGAAGGGGAGAGTGGAATTCCATGTGTAGCGGTG  697

Query  673  AAATGCGTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGTCTGTAACTGA  732
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  698  AAATGCGTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGTCTGTAACTGA  757

Query  733  CGCTGAGGCTCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGT  792
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  758  CGCTGAGGCTCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGT  817

Query  793  AAACGATGAGTGCTAGGTGTTGGATCCTTTCCGG-ATT  829
            |||||||||||||||||||||||||||||||||| |||
Sbjct  818  AAACGATGAGTGCTAGGTGTTGGATCCTTTCCGGGATT  855
```

BIOGRAPHY

Miss Yupawadee Chaodong was born on November 13, 1985 in Buriram, Thailand. She graduated with Bachelor degree of Science in Biotechnology from the Faculty of Agricultural Technology at Walailak University in 2008.