London Metropolitan University

Enumeration, Isolation, Identification and Probiotic Characterisation of Lactic Acid Bacteria from Nigerian Human Breast Milk

This thesis is submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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By

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DECLARATION

I hereby declare that the work reported in this thesis was carried out by me under the careful supervision of Dr. Hamid B. Ghoddusi, Prof. Jane Sutherland, Dr Brigitte Awamaria and Dr. Amara Anyogu. The works of other researchers cited in this thesis were duly acknowledged.

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DEDICATION

This thesis is dedicated to my family who had sacrificed immensely towards the realisation of this PhD degree.

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ABSTRACT

The aims of the research were to investigate the population and diversity of lactic acid bacteria (LAB) in human breast milk, to assess the probiotic potential of the identified LAB, to examine the antimicrobial resistance profile of the LAB and to investigate the possible relationship between the identified bacterial population and diversity with stage of lactation, number of children and diet. Samples of breast milk were collected from 20 breast feeding mothers. Standard spread plating on MRS agar and MRS agar supplemented with L-cysteine HCl (MRS-cys) was used for the enumeration of the organisms. Phenotypic identification of 108 recovered isolates was carried out. All isolates were Gram positive and oxidase negative, some were catalase positive and others were catalase negative. Further identification was carried out by grouping the isolates using repetitive sequence based PCR (rep-PCR). A total of nineteen groups were generated from the rep-PCR DNA profiles. This was followed by genotypic identification using 16S rRNA gene sequencing. The 16S rRNA gene sequencing identified LAB and non-LAB. Most of the LAB isolates belonged to the *Lactobacillus* genus, the rest were *Leuconostoc, Weissella, Streptococcus* and *Enterococcus* spp. The non-LAB were *Staphylococcus epidermidis* and *Staphylococcus hominis.* To examine the potential probiotic characteristics of the isolates, at least one representative isolate from each species of LAB (11 in total) were selected and characterised through a series of experiments. Initially the acid and bile resistance of the selected isolates were studied. All eleven LAB showed good tolerance to low pH and high concentration of bile salt. The antimicrobial activity of the isolates was also assessed. Again all eleven LAB produced antibacterial metabolites that inhibited

the growth of all the four indicator bacteria (*Bacillus cereus, Escherichia coli, Salmonella Enteritidis* and *Staphylococcus aureus*) in both unbuffered and buffered agar spot tests. The antimicrobial activity of the LAB using Agar well diffusion assay revealed antimicrobial properties of some of the studied LAB. The ability of the eleven selected LAB to produce exopolysaccharide, deconjugate bile salt and reduce cholesterol was investigated. The entire eleven LAB produced exopolysaccharide, deconjugate bile salt and reduce cholesterol. The phenotypic antimicrobial resistance profile of the eleven LAB was assessed. Furthermore, the genetic background of the phenotypic resistance was also investigated. All eleven LAB were sensitive to ciprofloxacin, gatifloxacin and quinupristin/dalfopristin and resistant to daptomycin. Most of the LAB were resistant to erythromycin, gentamicin tetracycline and vancomycin. Antimicrobial resistance genes for erythromycin and tetracycline were confirmed for three LAB.

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Abbreviation

°C= degrees Centigrade

AMR = antimicrobial resistance

BLAST = Basic Local Alignment Search Tool

BSAC = British Society for Antimicrobial Chemotherapy

BSH = Bile salt hydrolase

cfu = colony forming unit

CLSI = Clinical and Laboratory Standards Institute

CO_2 = carbon dioxide

DCs = dendritic cells

DNA = deoxyribonucleic acid

dNTP = deoxyribonucleotide triphosphate

e.g = example

EFSA= European Food Safety Authority

EPS = exopolysaccharide

et al = and others

EUCAST = European Committee on Antimicrobial Susceptibility testing

FAME = fatty acid methyl ester

FAO = Food and Agricultural Organisation

g = Gram

GC = gas chromatography

GIT = gastrointestinal tract

GRAS = generally recognized as safe

h = hour

H_2O_2 = hydrogen peroxide

HAMLET = human alpha-lactalbumin made lethal to tumour cells

HCl = hydrochloric acid

HMOs = human milk oligosaccharides

L=litre

LAB = lactic acid bacteria

LAB = lactic acid bacteria

LDL = low density lipoprotein

MALDI-TOF-MS = Matrix Assisted Laser Desorption Ionization-Time-Of-Flight Mass Spectrometry

Mg = milligram

MIC= Minimal Inhibitory Concentration

min = minute

ml = millilitres

MLN = mesenteric lymph nodes

MRD = Maximum Recovery Diluent

MRS agar = de Man Rogosa Sharpe agar

MRS-cys agar = de Man Rogosa Sharpe and cysteine agar

NaOH = sodium hydroxide

NCBI = National Center for Biotechnology Information

PBS = Phosphate Buffered Saline

PCR = polymerase chain reaction

PCR-DGGE = PCR-Denaturing Gradient Gel Electrophoresis

qPCR = quantitative polymerase chain reaction

QPS = Qualified Presumption of Safety

RAPD = random amplified polymorphic DNA

rep-PCR = repetitive element palindromic polymerase chain reaction

rpm = revolution per minute

rRNA = ribosomal ribonucleic acid

RT-qPCR = Quantitative Real Time PCR

s = second

SCFAs = short-chain fatty acids

sp = species (singular)

spp = species (plural)

subsp. = sub-species

TBE = trisborate ethylene diamine tetra acetic acid

TDCA = taurodeoxycholic acid

TSB = tryptone soya broth

USFDA = United States Food and Drug Administration

v/v = volume per volume

w/v = weight per volume

WHO = World Health Organisation

μg = micrograms

μg/ml = microgram per mililiters

μl= microliters

CHAPTER ONE: LITERATURE REVIEW

1.1 Introduction

Breast milk is an important food and nourishment for both new born babies and infants. Besides its nutritional composition, it also contains exosomes, immunoglobulins and microRNA that promote the development of babies' immune systems (Admyre *et al*. 2007). Micro RNA (miRNA) has been shown to be a transferable genetic material passed on from mother to child. It is suggested that miRNA are contained within microvesicles or exosomes (Kosaka *et al*. 2010). A substance also found in human breast milk, human alpha-lactalbumin made lethal to tumour cells (HAMLET), has been shown to destroy over 50 different kinds of cancer cells (Mossberg *et al*. 2010). Importantly, human breast milk has also been shown to contain some beneficial bacteria such as bifidobacteria and lactic acid bacteria (LAB) that help in developing a healthy microbiota in the gut of babies. Some of these beneficial bacteria have been shown to have probiotic potential. Numerous health benefits have been associated with the ingestion of probiotics and these include: improving the immune system, preventing intestinal infection especially diarrhoea, reducing allergy problems as well as improving recovery after antibiotic therapy. (Gueimonde *et al*. 2007, Martin *et al*. 2009 and Arboleye *et al*. 2011). These diverse components of human breast milk therefore promote the health, growth, development and overall wellbeing of babies and infants.

Breast milk contains complex carbohydrates called human milk oligosaccharides (HMOs). There are more than two hundred HMOs in breast milk but despite their carbohydrate composition, they do not provide nourishment for babies, but

instead are utilised by bacteria, especially enhance growth of bifidobacteria and LAB that are associated with health benefits (Gueimonde *et al*. 2007, Gibson and Roberfroid, 2008). Utilisation of HMOs as a substrate for growth of bacteria encourages rapid proliferation of these organisms, encouraging favourable competition and contributing to eradication of pathogenic microorganisms (Gibson and Roberfroid, 2008 and Petherick, 2010). Human milk oligosaccharides can also prevent the binding of *Campylobacter jejuni*, a pathogenic bacterium that is associated with infant diarrhoea, to the intestinal epithelial cells.

Furthermore, Zivkovic *et al*. (2011) have demonstrated that many oligosaccharides and glycoconjugates in human breast milk inhibit binding of pathogenic bacteria and toxins by acting as decoys. Anti-adhesive activity of HMOs against *Streptococcus pneumoniae*, enteropathogenic *Escherichia coli*, *Listeria monocytogenes* and *Vibrio cholerae* has also been revealed by Zivkovic *et al*. (2011). Breast-feeding is therefore an important source of LAB in the infant gut.

1.2 The concept of probiotics

Probiotics are viable microorganisms with proven health benefit that can be present in human and animal milk or fermented milk products which, when consumed, promote the health and well-being of the consumer. Probiotics have also been defined by the World Health Organisation (WHO) as “living microorganisms which when administered in adequate amounts confer health benefit on the host (FAO/WHO, 2001). Potentially beneficial microorganisms

include some species of bacteria and yeast including bifidobacteria and LAB, especially *Lactobacillus gasseri, Lactobacillus rhamnosus, Lactobacillus paracasei, Lactobacillus acidophilus* and *Lactobacillus plantarum* (Suganya *et al*. 2013 and Khedid *et al*. 2009), *Pedicoccus pentosaceus* (Gerez *et al*. 2006), *Weisella* species, *Bifidobacterium infantis*, *Bifidobacterium breve*, *Bifidobacterium longum* and *Bifidobacterium adolescentis* (Matsuki *et al*. 1999 and Onyibe *et al*. 2013). These bacteria can have a significant impact on the immunological, digestive and respiratory systems and thus prevent and relieve infectious diseases in children (FAO/WHO, 2006). The clinical applications of probiotics incude: treatment of diarrhoeal diseases associated with antibiotic therapy and travellers' diarrhoea, irritable bowel syndrome, allergies and eradication of *Helicobacter pylori* that causes intestinal ulcer and gastritis (Hamilton-Miller, 2003). The criteria for selecting probiotic strains for use as supplements according to Plummer *et al*. (2004), Jennifer *et al*. (2005) and FAO/WHO (2006) are:

(1) The organisms should be non-pathogenic
(2) The organisms should maintain viability during production of a supplement
(3) The organisms should maintain viability during passage through the acidic environment of the stomach
(4) The organisms must be genetically stable
(5) They must be tolerant to bile salts
(6) They should produce bile salt hydrolase enzyme

(7) They should have the ability to produce antimicrobial compounds that will inhibit the proliferation of known pathogens

(8) They should have the ability to adhere to the gut epithelial tissues

(9) They should have a positive effect on the immune system

(10) They should have a proven clinical research record

1.3 Origin of bifidobacteria and lactic acid bacteria in human breast milk

It was thought that potentially beneficial bifidobacteria and LAB originate from the mammary gland, maternal nipples and the immediate surroundings of the breast skin. However, some studies have indicated that the origin of viable beneficial bacteria in human breast milk is the maternal gut (Perez *et al.* 2007 and Jeurink *et al*. 2013). Immune cells (CD18+), dendritic cells and macrophages in the mother's gut pick up beneficial bacteria and transport them to the mammary gland using the lymphatic system (Jeurink *et al*. 2013). It has been shown that dendritic cells (DCs) can open the tight junctions between intestinal epithelial cells and penetrate the gut epithelium with their dendrites, enabling DCs to pick commensal bacteria directly from the gut lumen without damaging the integrity of the epithelial barrier (Martín *et al.* 2004. Perez *et al.* 2007 and Fernandenz *et al.* 2013). This process has been likened to a *Salmonella typhimurium* strain that, although it was deficient in invasion genes, was able to reach the spleen alive after oral administration to mice (Rescigno *et al.* 2001). Macrophages have also been shown to be capable of disseminating non-invasive bacteria (Jeurink *et al*. 2013). In research comparing bacterial diversity of breast milk and breast skin, major genotypic differences were found among the lactobacilli, enterococci and

bifidobacteria from the different environments (Martin *et al.* 2003 and Gueimonde *et al.* 2007). Logical arguments have been established ruling out presence of bacteria in human milk as a result of contamination and excluding the infant's mouth, or mother's breast skin, as vehicles transmitting bacteria to human breast milk. It has been shown that live beneficial bacteria orally administered to lactating women in a capsule can be retrieved from their milk (Jimenez *et al.* 2008 and Arroyo *et al.* 2010). Moreover, studies in mice have shown that bacteria from the gut translocate to mesenteric lymph nodes (MLN) and mammary glands during the late stage of pregnancy and the beginning of lactation (Fernandez *et al.* 2004 and Jeurink *et al.* 2013). The migration process from the maternal gut to the mammary gland is influenced by the ability of bacteria to adhere to mucus. Production of exopolysaccharides (EPS) by bacteria enhances their survival during systemic transportation. Research by Fanning *et al.* (2012) has shown that *Bifidobacterium breve* strain UCC2003 produces an EPS thought to facilitate ability of the bacterium to remain immunologically silent by escaping the adaptive B-cell host response. Potentially beneficial bacteria such as LAB and bifidobacteria present in milk may have an endogenous origin and may not be the result of contamination from the surrounding breast skin (Martin *et al.* 2003).

1.4 Lactic acid bacteria

Lactic acid bacteria are a group of bacteria characterized by diverse morphological, physiological and metabolic features. They are Gram positive, anaerobic or facultatively anaerobic, non-sporing cocci or rod and are catalase and oxidase negative. They produce lactic acid as the primary end product of

carbohydrate fermentation (Salminen and Wright, 1998, Khedid *et al*. 2009, Suganya *et al*. 2013 and Tusar *et al*. 2014). The genera of LAB consist of *Lactobacillus, Lactococcus, Lactosphaera, Leuconostoc, Enterococcus, Aerococcus, Alloiococcus, Carnobacterium, Globicatella, Dolosigranulum, Oenococcus, Vagococcus, Streptococcus, Weisella, Pediococcus* and *Tetragenococcus*. Classification of these bacteria into genera before the advent of DNA (molecular) identification was based on morphology, glucose fermentation, temperature of growth, ability to grow in high sodium chloride and organic and inorganic acids concentration, as well as lactic acid production. LAB can utilise glucose in two ways: by glycolysis (Embden-Meyerhof pathway) or the 6-phosphogluconate and phosphoketolase pathway. Glycolysis results in lactic acid as the main end product (homo-lactic fermentation), while the 6-phosphogluconate and phosphoketolase pathway results in multiple end products including lactic acid, ethanol, carbon dioxide and acetic acid (hetero-lactic fermentation). Therefore, LAB are phenotypically classified into two groups: homo-fermentative and hetero-fermentative (Salminen and Wright, 1998, Ammor *et al*. 2006 and Suskovic *et al*. 2010).

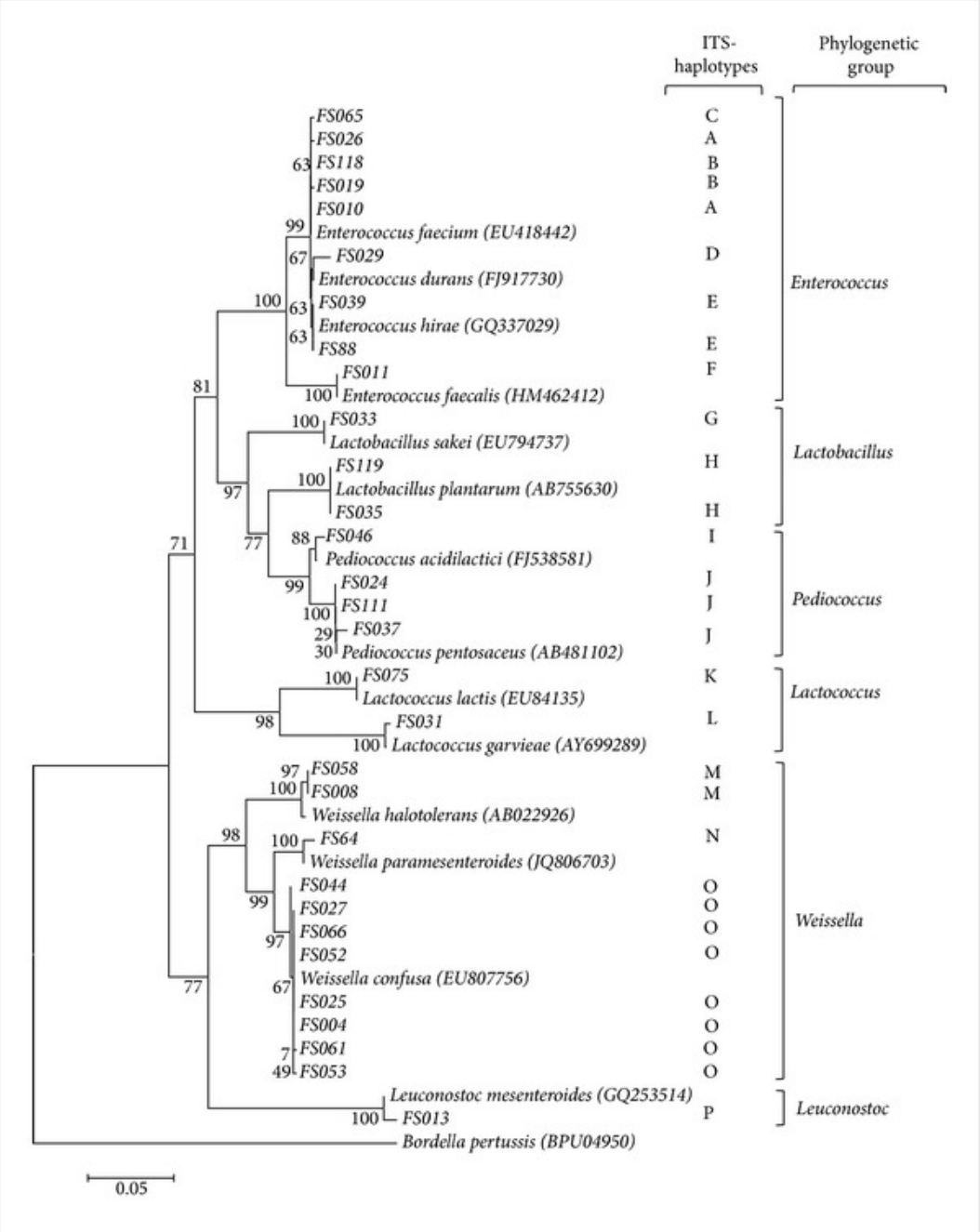

Figure 1.1: Phylogenetic tree of some lactic acid bacterial isolates based on 16S rDNA partial sequences, using the neighbor-joining method. Bordetella pertussis was used as an out group. Fhoula *et al*. (2013).

1.5 Production of antimicrobial substances and adhesion inhibitors by lactic acid bacteria

Lactic acid bacteria produce a variety of antimicrobial organic acids that kill or inhibit growth of pathogenic microorganisms. Homo and hetero-fermentation of glucose yield potent end products such as lactic acid, acetic acid, ethanol and carbon dioxide. Carbon dioxide manifests antimicrobial properties in two ways: firstly, CO_2 is itself toxic to some microorganisms, and secondly, creation of an anaerobic atmosphere prevents proliferation of aerobic microorganisms, especially concentrations of 85% nitrogen, 10% hydrogen and 5% carbon dioxide (Singleton, 2004). Furthermore, LAB produce hydrogen peroxide (H_2O_2) which has a strong oxidizing effect on bacterial cells resulting in destruction or inactivation of pathogenic microorganisms (Suskovic *et al*. 2010). Moreover, bacteriocins are antimicrobial substances produced by some LAB, including *Lactobacillus sake, Lactobacillus delbrueckii, Lactobacillus helveticus, Lactobacillus acidophilus, Lactobacillus reuteri, Lactobacillus plantarum, Lactococcuslactis, Lactococcus plantarum, Lactococcus cremoris, Streptococcus mutans, Pediococcus acidilactici* and *Leuconostoc mesenteroides* subsp. *mesenteroides* (Ammor *et al*. 2006). For example, nisin and diplococcin are bacteriocins produced by *Lactococcus*. Nisin inhibits growth of some Gram positive bacteria, such as *Clostridium botulinum* and *Listeria monocytogenes* (Ammor *et al*. 2006 and Suskovic *et al*. 2010). Other bacteriocins include lacticin, lactocin, mutacin, carnocin, salvaricin and streptococcin (Ammor *et al*. 2006 and Suskovic *et al*. 2010).

Apart from these, LAB produce adhesion inhibitors that prevent binding of pathogenic microorganisms to the intestinal epithelial cell of a host and thus reduce ability of the pathogen to colonize the intestine (Zivkovic *et al*. 2011). Adhesion of microbes to the surface of the intestinal tract is important for stability of microorganisms in the intestinal environment because if they cannot adhere, they will be flushed away as a result of fluid secretions and peristaltic movement.

1.6 Identification of microorganisms

In the past, identification of microorganisms was based on biochemical and physiological (phenotypic) characteristics of the isolates such as Gram staining, presence of certain enzymes (catalase, oxidase, coagulase, protease, lipase and lecithinase), glucose fermentation, motility, spore formation and morphological features. However, while phenotypic characteristics are still valuable, advances in molecular biology have resulted in rapid and reliable methods of identifying microbes, particularly application of genetic sequencing to identify bacteria to species and even strain level. There are three types of ribosomal ribonucleic acid (rRNA) 5SrRNA, 16SrRNA and 23SrRNA but 16SrRNA provides the ideal balance between information content (Baker *et al*. 2011). This evolutionary chronometer (16SrRNA) is used for analysing phylogenetic relationships between bacteria with different sequences (Baker *et al*. 2011). Fragments of DNA containing 16SrRNA are generated by the polymerase chain reaction (PCR). These primers have been designed to anneal to the conserved regions of genes and thus facilitate use of one universal primer set to amplify 16SrRNA from phylogenetically different bacteria (Nicholl, 2008, Baker *et al*. 2011 and Rahman

et al. 2012). If 16SrRNA gene sequencing is not able to differentiate closely related species, then sequencing of the pheS, rpoA and rpoB genes is recommended (Anyogu, *et al*. 2014).

The first discovery of repetitive extragenic palindromic (REP) element was observed in the genomes of *Escherichia coli* and *Salmonella typhimurium* (Higgins *et al*. 1982 and Stern *et al*. 1984). Repetitive element sequence based-polymerase chain reaction (rep-PCR) could be defined as the process of using known primers for the PCR amplification of interspersed repetitive DNA elements of bacterial and fungal genomes (Gillings and Holley, 1997). The amplified DNA fragments of rep-PCR after separation by gel electrophoresis generate a genomic fingerprint that can be used for subspecies and strain description (Gillings and Holley, 1997 and Healy *et al*. 2005).

The EzTaxon database is a recently developed quality-controlled 16S rRNA gene sequence database for the identification of bacteria. It is the most effective database for the identification of 16S rRNA gene sequences (Kim *et al*. 2012). The 16S rRNA gene is the most preferred method for the molecular identification of microorganisms but the limitation of the 16S rRNA gene sequencing is the difficulty in interpretation of the nucleotide sequences of the DNA. Moreover, the GenBank which is also used for the identification of bacterial DNA sequence that is searched using the National Center for Biotechnology Information Basic Local Alignment Search Tool (NCBI BLAST), have been shown to lack peer-reviewed sequences of type strains and sequences of non-type strains (Park *et al*. 2012 and Yoon *et al*. 2017). EzTaxon is therefore more discriminative and reliable. BIBI

database is also a new database that has also been developed for the interpretation and identification of the bacterial 16S rRNA gene sequences (Park *et al*. 2012). It is recommended that 16S rRNA gene sequencing results should be evaluated by Genbank and confirmed by EzTaxon or other quality-controlled databases (Kim *et al*. 2012, Park *et al*. 2012 and Yoon *et al*. 2017).

Microorganisms can also be identified by the type of fatty acids they produce, using fatty acid methyl ester analysis (FAME analysis). In this technique, the lipids of a pure culture are extracted, esterified and quantified by gas chromatography (GC). The GC profile is then compared by computer with profiles of other microbes grown in identical conditions. Although FAME analysis is rapid and inexpensive, interpretation of the results can be difficult (Baker *et al*. 2011).

Identification of LAB using phenotypic and genotypic analysis has been undertaken by many researchers including; Martin *et al*. (2009) Rahman *et al.* (2012) and Kavitha and Davasena, (2013). Molecular studies on human breast milk based on 16S rRNA genes have revealed the presence of bifidobacteria including *Bifidobacterium breve* and *Bifidobacterium longum* subsp. *infantis*, *Lactobacillus rhamnosus* and *Lactobacillus casei*, detected by PCR-DGGE (PCR-Denaturing Gradient Gel Electrophoresis) and RT-qPCR (Quantitative Real Time PCR), whole genome shotgun sequencing as well as16S rRNA gene sequencing (Martin *et al*. 2009, Rahman *et al.* 2012 and Kavitha and Davasena, 2013). Use of culture-dependent molecular techniques, especially those based on 16S rRNA gene sequencing has demonstrated the biodiversity of human milk microbiota.

Therefore, confirming presence of *Staphylococcus*, LAB and *Bifidobacterium* in human breast milk (Martin *et al*. 2003, Martin *et al*. 2007, Albesharat *et al*. 2011, Makino *et al*. 2011, Fernandez *et al*. 2013 and Tusar *et al*. 2014).

1.7 Production of exopolysaccharides by lactic acid bacteria

Exopolysaccharides (EPSs) are biopolymers secreted by bacteria. They protect bacterial cells from toxic substances, osmotic stress, dessication and bacteriophage. Moreover, they also facilitate adhesion of bacterial cells to surfaces (Hongpattarakere *et al*. 2011). The rheological properties of lactic acid bacterial exopolysaccharides are useful in the production of fermented dairy products and other fermented foods (Sasikumar *et al*. 2017). Beside the gelling properties of lactic acid bacterial exopolysaccharides, they also possess health promoting properties. The health promoting attributes of lactic acid bacterial exopolysaccharides include; cholesterol reduction, antimicrobial activity, antioxidant activity, antitumor, antiulcer and immunomodulation (Hongpattarakere *et al*. 2011, Ismail and Nampoothiri, 2013, Joshi and Koijam, 2014, Domingos-Lopes *et al*. 2017, Sasikumar *et al*. 2017 and Riaz-Rajoka *et al*. 2018). Furthermore, lactic acid bacterial exopolysaccharides are stable in gastrointestinal tract which facilitate colonisation of beneficial LAB in the gut (Riaz-Rajoka *et al*. 2018).

LAB produce various kinds of EPSs which differ in chemical compostion and structure (Ruas-Madiedo *et al*. 2002). Some *Leuconostoc mesenteroides* subsp. *mesenteroides* and *Lactobacillus fermentum* produce dextrans which are α D-glucans, *Streptococcus salivarius* subsp. *thermophilus* and *Lactobacillus reuteri*

produces fructans which are levans (Ruas-Madiedo *et al*. 2002 and Badel *et al*. 2011). Both dextran and fructan belong to the homopolysaccharides group of exopolysaccharides (Badel *et al*. 2011). On the other hand, *Lactobacillus pentosus, Lactobacillus plantarum* and *Lactobacillus rhmnosus* produce glucose and galactose. These sugars belong to the heteropolysaccharides group of exopolysaccharides (Badel *et al*. 2011).

Additionally, some lactic acid bacterial exopolysaccharides are prebiotic. Prebiotics are non digestible oligosaccharides that stimulate growth of some beneficial bacteria in the gut (Hongpattarakere *et al*., 2011 and Badel *et al*., 2011). Hongpattarakere *et al*., 2011 reported the prebiotic property of EPS produced by some lactic acid bacteria especially *Weissella cibaria* A2 which exhibited bifidogenic effect.

The antidiabetic and cholesterol lowering properties of exopolysaccharides produced by *Lactobacillus plantarum* BR2 were reported by Sasikumar *et al*. (2017), the EPS was not toxic to normal cells. Similarly, Ismail and Nampoothiri, 2013 have studied the antioxidant and antitumor of EPS produced by *Lactobacillus plantarum* MTCC 9510. The EPS was found to have antitumor properties and also non toxic to normal cells.

1.8 Bile salt hydrolase activity of lactic acid bacteria

Bile salt hydrolase also referred to as cholylglycine hydrolase are enzymes that hydrolyse the amide bond of conjugated bile salts, thereby releasing the amino acid moiety from the steroid core and produce deconjugated bile salts (Begley *et al*. 2006 and Allain *et al*. 2018). Ability of lactic acid bacteria to produce bile

hydrolase enzyme is an important criterion in selection of probiotic bacteria (FAO/WHO, 2002 and Begley *et al*. 2006). Bile is synthesised in the liver, it is composed of bile acids, cholesterol, phospholipids, and biliverdin (Begley *et al.* 2006). Cholic and chenodeoxycholic acids are primary bile salts which are conjugated bile salts. Bile salt hydrolase (BSH) enzyme facilitates deconjugation of cholic and chenodeoxycholic acids to unconjugated bile salts and glycine or taurine residue (Begley *et al*. 2006). Deconjugated bile acids occuring from bile salt hydrolysis have more inhibitory effects on bacteria than conjugated bile acids. Therefore, BSH plays a significant role in tolerance of some LAB to bile and bile salts. Deconjugated bile salts are less absorbed in the human intestine, therefore facilitating the excretion of free bile acids through faeces (Begley *et al*. 2006). Although, excessive excretions of bile salts could decrease the total amount of bile salts in human bodies. But lost bile salts could be restored by *de novo* synthesis from cholesterol which could reduce the level of serum cholesterol in human body (Begley *et al.* 2006).

It has been reported that BSH facilitate assimilation of cholesterol into lactic acid bacterial cell membranes (Pereira *et al*. 2003 and Taranto *et al*. 2003). BSH activity is therefore important in selection of LAB with cholesterol lowering properties, as non deconjugating LAB do not assimilate cholesterol *in vitro* (Kumar *et al*. 2012 and Anandharaj and Sivasankari, 2014).

Moreover, BSH detoxify bile salts and thus, increase survival of producing strains in the intestine (Kumar *et al*. 2012 and Allain *et al*. 2018). BSH activity of

beneficial lactic acid bacteria is therefore, vital in facilitating their survival in the harsh environmental condition of the gastrointestinal tract (Begley *et al*. 2006).

BSH activity is a natural process that aids in reduction of cholesterol (Allain *et al*. 2018). However, because deconjugated bile salts are less efficient than conjugated bile salts in the emulsification of dietary lipids, therefore, BSH activity could affect normal lipid digestion. Thus, the absorption of fatty acids could be impaired (Begley *et al* 2006).

The BSH activity of *Lactobacillus pentosus* and *Lactobacillus plantarum* was reported by saraniya and Jeevaratnam (2015). Similarly, cholesterol assimilation of *Lactobacillus pentosus* and *Lactobacillus plantarum* was also reported by Saraniya and Jeevaratnam (2015). The ability of *Lactobacillus plantarum* to produce BSH enzyme and assimilate cholesterol was also reported by Yadav *et al*. (2016).

1.9 Cholesterol assimilation by LAB

Hypercholesterolemia is associated with cardiovascular diseases which causes ill health and sudden death in both developed and developing countries. Manson *et al*. (1992) reported that 1% reduction in serum cholesterol decrease risk of coronary heart disease by 2–3%. Undesirable side effects of conventional drug therapy for hypercholesterolemia have prompted research into alternative safe therapy. Studies on cholesterol assimilation of LAB have demonstrated their ability to reduce cholesterol *in vitro* and *in vivo* (Pereira *et al*. 2003, Begley *et al*. 2006, Damodharan *et al*. 2015, Saraniya and Jeevaratnam 2015, Shehata *et al*. 2016 and Yadav *et al*. 2016).

Oral administration of some probiotics *in vivo* has been reported to reduce cholesterol level by about 22 to 33% (Pereira and Gibson 2002). Similarly, hyperlipidemic patients that were given *Lactobacillus sporogenes* for 90 days showed a 32% and 35% decrease in their LDL and total cholesterol levels, respectively (Mohan *et al*. 1990). In another human study carried out by Lin *et al*. (1989), blood cholesterol was significantly reduced in volunteers who were given tablets of *Lactobacillus bulgaricus* and *Lactobacillus acidophilus* for 16 weeks every day. Many studies on cholesterol-reducing potential of probiotics have not elucidated the mechanism of cholesterol assimilation by probiotics. However, cholesterol-lowering properties of LAB have been attributed to BSH activity, cholesterol conversion to coprostanol (Coprosterol), production of short-chain fatty acids (SCFAs) during growth of probiotics and binding of cholesterol to bacterial cell wall, incorporation of cholesterol into the cellular membrane and coprecipitation of cholesterol with deconjugated bile (Liong and Shah 2005, Kumar *et al*. 2012, Tsai *et al*. 2014, Tomaro- Duchesneau *et al*. 2014 and Shehata *et al*. 2016). The hypocholesterolemic property of some probiotics could be strain-specific (Tsai *et al*. 2014). Moreover, reduction of cholesterol *in vitro* has been shown to be higher in the presence of bile salt in MRS broth (Tahri *et al*. 1997 and Praveen *et al*. 2007). Furthermore, cholesterol assimilation potential of some *Lactobacillus* species is related to their ability to produce BSH enzymes (Anandharaj and Sivasankari, 2014).

1.10 Antimicrobial resistance of LAB

Resistance of some bacteria to antimicrobials is a global public health risk. Antibiotic resistance could be intrinsic (natural) or acquired. Intrinsic resistance is an inherent characteristic of some LAB and this could be attributed to presence of low-affinity targets, low cell permeability, antimicrobial inactivation of the antibiotics and the presence of efflux mechanisms (Ammor *et al*. 2008). However, intrinsic resistance and resistance by mutation are rarely disseminated (Ammor *et al*. 2008). Acquired resistance involves horizontal transfer of genes, especially those carried on mobile genetic elements (Florez *et al*. 2016). Many antimicrobial resistance (AMR) genes from both pathogens and commensal bacteria are similar and carry transferable genetic elements. Commensal bacteria such as LAB are therefore likely to disseminate AMR genes in the gut (Zhang *et al*. 2011). Potential transferability of antimicrobial resistance genes by LAB to other bacteria is inevitable. This could therefore, promote dissemination of AMR genes amongst different strains and species of bacteria (Ouoba *et al*. 2008 and Florez *et al*. 2016). For safety reasons, all probiotic bacteria must be screened for antimicrobial resistance and resistance genes (FAO/WHO 2006). Some antimicrobials used in the determination of antimicrobial resistance with their mechanism of action and resistance (Ouoba *et al*. 2008, Devirgiliis *et al*. 2013 and Guo *et al*. 2017) are shown in Table 1.1.

Table 1.1 Some antimicrobials used in the determination of antimicrobial resistance with their mechanism of action and resistance.

Pharmacological group	**Antimicrobial and resistance gene**	**Mechanism of action**	**Mechanisms of resistance**
Aminoglycosides	Amikacin Apramycin Gentamycin (*aac*(6″),*aph* (2″), *aac*(3″) V, *ant*(2″)-*1*) Kanamycin (*aph*(3”)-*1*,*aph*(3”)-*111*, *ant*(2”)-*I*) Neomycin (*aph*(3”)-*11aph*(3”)-*11*, *aph*(3”)-*111)* Spectinomycin Streptomycin (*str*A, s*tr*B, *aad*A, *aad*E)	Ribosome	Enzymatic inactivation Modification of cell permeability Target site mutations (alterations at the ribosomal binding sites)
βeta-lactams	Amoxicillin Ampicillin (*blaz*A) Imipenem Oxacillin Penicillin (*blaz*A) Cloxacillin	Cell wall	Efflux Enzymatic inactivation (β-lactamase) Modification of cell permeability Target site mutations (altered penicillin-binding proteins)
Chloramphenicol	Chloramphenicol (*cat*A)	Ribosome	Efflux Enzymatic inactivation (mainly acetylases, phosphotransferases) Target site mutations Modification of cell permeability
Glycopeptides	Linezolid (*cfr*) Vancomycin (*van*A, *van*B, *van*C, *van*E, *van*X)	Cell wall	Target site mutations (reduction of vancomycin binding affinity by substitution of a terminal D-lactate or D-serine for D-alanine)
Lincosamides	Clindamycin (*lnu*(A), *lnu*(B)) Lincomycin	Ribosome	Efflux Enzymatic inactivation Target site alterations (methylases)

Ouoba *et al*. (2008), Devirgiliis *et al*. (2013) and Guo *et al*. (2017)

Table 1.1 continued

Pharmacological group	**Antimicrobial and resistance gene**	**Mechanism of action**	**Mechanisms of resistance**
Macrolides	Erythromycin (*erm*(A), *erm*(B) and *erm*(C)) Roxithromycin	Ribosome	Efflux Enzymatic inactivation Target site alterations (methylases)
Quinolones	Ciprofloxacin (*gyr*A, *par*C) NalidixicAcid	DNA gyrase DNA topoisomerase	Efflux Lower target expression levels Modification of cell permeability Target site mutations
Rifamycins	Rifampin	RNA polymerase	Enzymatic inactivation Modification of cell permeability Target site mutations Target duplication
Sulfonamides	Sulphamethoxazole Trimethoprim (*dfr*A, *dfr*D)	Dihydropteroate Synthetase (DHPS)	Target site mutations Plasmid-borne alternative drug-resistant variants of DHPS
Streptogramins	Quinupristin/dalfopristin (*vatC, vatD, vatE*)	Ribosome	Efflux Enzymatic inactivation Ribosome Target site alterations (methylases)
Tetracyclines	Chlorotetracycline Tetracycline (*tet(*K), t*et*(L), *tet*(M), *tet*(O), *tet*(S), *tet*(W))	Ribosome	Efflux Enzymatic inactivation Target protection

Ouoba *et al*. (2008), Devirgiliis *et al*. (2013) and Guo *et al*. (2017)

1.11 Antimicrobial resistance of LAB isolated from human breast milk

Antimicrobial resistance of some LAB has been attributed to misuse of antimicrobials in food chain particularly in animal production, aquaculture and agriculture (Egervan *et al*. 2009). Once AMR LAB gets into the gut, they may function as reservoirs of mobile AMR genes that could potentially be transferred to other commensal and pathogenic bacteria (Ouoba *et al*. 2008 and Karapetkov *et al*. 2011). Some studies have suggested that LAB present in human breast milk originate from maternal gut as a result of translocation to maternal mammary gland (Perez *et al*. 2007, Fernandenz *et al*. 2013 and Jeurink *et al*. 2013).

Lactobacillus oris HMI68 isolated from human breast milk was reported to have multidrug resistance to antimicrobials. It exhibited phenotypic resistance to nine antimicrobials including ampicillin and gentimicin although it was sensitive to kanamycin and novobiocin (Anandharaj and Sivasankari, (2014). Similarly, multidrug resistance resistance in *Enterococcus faecium* isolated from human breast milk to ciprofloxacin, ampicillin, gentamicin, penicillin and vancomycin was reported by Kivanc *et al*. (2016) and Reis *et al*. (2016). The presence of *tet*(W) gene in *Lactobacillus plantarum* and *Lactobacillus reuteri* isolated from human breast milk was reported by Egervin *et al*. (2009). Furthermore, *tet*(M), *erm*(B) and *sul2* genes were detected from *Lactobacillus plantarum* and *Lactobacillus reuteri* isolated from human breast milk (Zhang *et al*.2011). However, Human alpha-lactalbumin made lethal to tumour cells (HAMLET) is a substance found in breast milk that has anti-tumour and bactericidal properties. It is an antimicrobial adjuvant that enhances biological activity of antibiotics when used simultaneously (Hakansson *et al*. 2011, Marks *et al*. 2012 and Marks *et al*.

2013). HAMLET has been shown to have antimicrobial potentiating effect on staphylococci and streptococci as well as numerous bacterial species with multi-drug resistance (Marks *et al*. 2013). Marks *et al*. (2013) studied potentiating effect of HAMLET on antibiotics including erythromycin, gentamicin, methicillin and vancomycin. They found out that HAMLET had an effective antimicrobial adjuvant thus, increased the efficacy of the antimicrobials. Drug resistant *Staphylococcus aureus* and *Streptococcus pneumoniae* were able to become sensitive to the antimicrobials in *in vitro* assays (MICs and MBCs) and for elimination of biofilms and nasopharyngeal colonisation *in vivo*.

Human breast milk is therefore endowed with various protective substances such as antibodies, HAMLET, immune stimulating properties, lysozyme, lactoferrin, oligosaccharides and other substances that have not yet been explored. These protective substances especially HAMLET has potential of reversing multiple drug resistance bacteria to susceptible bacteria when used in combination with antimicrobials. Breast fed babies and infants could consequently respond effectively to antimicrobial treatments.

The specific aims of this research were:

1. To investigate the population and diversity of LAB in Nigerian human breast milk.

2. To investigate the probiotic potentials of the isolates.

3. To investigate the phenotypic antimicrobial resistance profiles of LAB and genetic background of phenotypic resistances.

4. To investigate the possible relationship between the identified bacterial population and diversity with donors' age, stage of lactation, gender of lactating babies, number of children and diet.

The main objectives of this research were:

1. To enumerate LAB recovered from Nigerian human breast milk.
2. To isolate potential LAB colonies.
3. To phenotypically identify the isolates.
4. To differentiate and group the isolates using rep-PCR.
5. To identify isolates to species level by sequencing the 16S rRNA gene.
6. To investigate the probiotic potential of the species by *in vitro* techniques, including acid and bile resistance, antimicrobial activity, cholesterol assimilation, bile salt hydrolase and exopolysaccharide production.
7. To investigate antimicrobial resistance profile and determination of resistance genes.
8. To undertake a survey on the background of breast milk donors to determine whether there is a relationship between the population and diversity of the LAB species recovered and stage of lactation, gender of lactating babies, number of children and diet.

Justification for the research

1. Although some research has been carried out on bifidobacteria in breast milk, few investigations have concerned LAB. With regard to Nigerian human breast milk, to the best knowledge of the author, no studies exist. It is therefore imperative to undertake research on LAB in order to provide information on their population and diversity as well as their probiotic potential.

2. To the best knowledge of the author, there has been very little research on the correlation between breast milk donors' demographics (stage of lactation, number of children, age and diet) and the diversity of LAB

Significance of the study

The outcome of this study will contribute to knowledge of the population and diversity of LAB in human breast milk. The research will explore the probiotic potential of selected LAB recovered from human Nigerian breast milk and this will promote use of beneficial LAB in biotherapy for treatment of diarrhoeal and other diseases in babies. Supplementation of milk formula and other baby foods with probiotic bacteria, especially in developing countries, could be advocated. This will benefit babies who could not be breast fed due to diseases associated with their mothers. Information derived from the research findings on how the population and diversity of LAB in human breast milk correlates with donors' demographics will provide information on factors that could increase or decrease the population or diversity of beneficial bacteria in human breast milk. Overall,

the findings of this research will benefit health policy makers, food industries, non-governmental agencies, the education sector and mothers.

CHAPTER TWO: ENUMERATION, ISOLATION AND IDENTIFICATION OF LACTIC ACID BACTERIA FROM HUMAN BREAST MILK

2.1 Introduction

Human breast milk is a balanced food for the nourishment of newly born babies. Besides its nutritional composition, it contains beneficial LAB that help in developing a healthy microbiota of the baby's gut (Fernandez *et al*. 2013 and Tusar *et al*. 2014). Some LAB found in human breast milk have protective effect against infant allergic and diarhoeal diseases. (Martin *et al*. 2015). Beneficial LAB present in human breast milk reduce incidence and severity of these illnesses by different mechanisms including; production of antimicrobial compounds and competitive exclusion (Fernandez *et al*. 2013 and Reis *et al*. 2016). According to Maldonado *et al*. (2012), the administration of *Lactobacillus fermentum* CECT5716 isolated from human breast milk to infants for a period of 6 months reduced the incidence rate of gastrointestinal infections, upper respiratory tract infections, and total number of infections to 46%, 27%, and 30% respectively.

A large and diverse population of beneficial bacteria in the infant gut has therefore been linked to good health in newly born babies and contributes to their healthy development in the future (Fernandez *et al*. 2013). Albesharat *et al*. (2011) support the hypothesis that suggest a vertical transfer of intestinal LAB from the mother's gut to the mammary gland and finally to the breast-fed infant's gut.

Investigators such as Martin *et al*. (2012) and Tusar *et al*. (2014) have demonstrated the presence of *Lactobacillus gasseri, Lactobacillus fermentum, Lactobacillus plantarum, Lactococcus lactis, Leuconostoc mesenteroides* subsp. *mesenteroides, Bifidobacterium breve, Bifidobacterium bifidum, Bifidobacterium*

longum, *Streptococcus salivarius*, *Enterococcus faecium*, *Enterococcus faecalis*, *Weissella confusa*, *Weissella cibaria*, *Staphylococcus epidermidis* and *Staphylococcus hominis* in human breast milk.

Early colonisation of the infant gut with potentially beneficial bacteria enables the lymphoid tissue associated with the gut to mature and consequently helps homeostasis of the intestinal epithelium tissue and contributes to regulation of the intestinal physiology (Matsuki *et al*. 1999 and Gueimonde *et al*. 2007). Breast fed infants have higher populations of *Bifidobacterium infantis* and other beneficial bacteria than infants who are fed formula milk (Matsuki *et al*. 1999 and Gueimonde *et al*. 2007). The formation of a stable gut microflora in human neonates is favourably influenced by dominance of a diverse population of beneficial bacteria and this is vital for healthy development of the infant gastrointestinal tract (GIT) and immune system (Arboleye *et al*. 2011).

The enumeration and isolation of bacterial cells is significant for the identification and characterization of bacteria. Various methods of genotypic identification of bacteria were used by Martin *et al*. (2003), Albesharat *et al*. (2011), Martin *et al*. (2012) and Tusar *et al*. (2014) to assess the composition of human breast milk microflora.

Albesharat *et al*. (2011) differentiated isolates from human breast milk isolated from 15 Syrian mothers using random amplified polymorphic DNA (RAPD). Isolates were identified as LAB by comparative 16S rDNA sequencing and Matrix Assisted Laser Desorption Ionization-Time-Of-Flight Mass Spectrometry (MALDI-TOF-MS) analyses. *Lactobacillus*, *Enterococcus*, *Streptococcus*,

Pediococcus and *Staphylococcus* were identified. RAPD and MALDI-TOF-MS patterns allowed comparison of the lactic microbiota on species and strain level in their study. Similarly, Tusar *et al*. (2014) also used RAPD to discriminate 86 presumptive isolates from human breast milk isolated from 11 Slovenian lactating mothers. Representatives of different RAPD groups were identified using 16S rDNA sequencing. Identified LAB were *Lactobacillus fermentum, Lactobacillus salivarius, Lactobacillus reuteri,* and *Enterococcus faecium*. *Bifidobacterium breve* species and *Staphylococcus epidermidis* were also identified. Martin *et al*. (2003) also used RAPD to discriminate isolates from human breast milk isolated from 8 Spanish breast feeding mothers. *Lactobacillus gasseri* and *Enterococcus faecium* were identified using 16S rDNA sequencing. However, Martin *et al*. (2012) identified *Staphylococcus*, *Lactobacillus*, and *Bifidobacterium* from 20 Spanish breast feeding mothers using Quantitative Real-Time PCR (qRTi-PCR), RAPD and 16S rRNA gene sequencing. Sakwanski *et al*. (2016) studied microbiota of human breast milk of Chinese lactating mothers using microbiota profiling based on the sequencing of fragments of 16S rRNA gene and quantitative polymerase chain reaction (qPCR). Breast milk samples were collected using standard protocol without aseptic cleansing and with aseptic cleansing of maternal nipples and breast surrounding. Presence of *Streptococcus* and *Staphylococcus* species for both collection protocols were reported. Sakwanski *et al*. (2016) also revealed a significantly higher number of bacteria identified in the “breastfeeding-associated microbiota” compared to milk obtained under aseptic conditions.

There is limited information regarding LAB present in Nigerian human breast milk. Many studies have focused on isolation of LAB from other continents of the world but not Africa. The aim of the study described in this chapter was to isolate and identify LAB from some samples of Nigerian human breast milk. This would enable selection of LAB isolates for further characterisation in regard to their probiotic properties and their antimicrobial resistance profile and antimicrobial resistance genes. The specific objectives of this study were:

- To enumerate and isolate LAB present in Nigerian human breast milk.
- To phenotypically identify the isolates based on cell and colony morphology and biochemical tests.
- To characterise and confirm the identity of isolates using molecular techniques such as rep-PCR and 16S rRNA gene sequencing.

2.2 Materials and methods

2.2.1 Donor recruitment

Breast milk samples were collected from 20 breast feeding mothers in Diamond Specialist Hospital Kaduna, Nigeria. Lactation period of donors was between 1 to 21 months. Ethics approval was granted by the London Metropolitan University Ethics Committee. Breast feeding mothers were contacted in person and the research explained to them. Those that showed interest were enlisted as participants. Donors were recruited after indicating their consent by signing the consent forms to voluntarily participate (Appendix 1).

2.2.2 Sample collection

The human breast milk samples were manually expressed into sterile tubes. Nipples of donors were not sterilised prior to milk collection. Milk samples were stored in an ice box at 1 to 2 °C but not frozen and transported to the microbiology laboratory of the Department of Food Technology, Kaduna Polytechnic, Nigeria. Samples were analysed as soon as possible within 6 h after collection. The media and diluent were prepared before collection of samples.

2.2.3 Enumeration of LAB

Breast milk samples were diluted ten-fold to 10^{-3} in maximum recovery diluent (MRD, CM0733, Oxoid, Basingstoke, UK). Volumes of 100 µl of the three dilutions and the undiluted milk sample were spread in duplicate on deMan Rogosa Sharpe agar (MRS, CM0361, Oxoid, UK) and in duplicate on MRS agar to which 0.5g/litre L- cysteine hydrochloride (C1276 Sigma, Gillingham, UK) had been added (MRS-cys agar). The plates were incubated anaerobically (in anaerobic jars (AG25, Oxoid, UK) with anaerogen pack (ANOO35A, Thermoscientific, UK) at 37 ^{o}C for 48-72 h. Colonies were counted, calculated and reported as cfu/ml.

2.2.4 Isolation of LAB isolates

Separated colonies were randomly picked from plates of MRS and MRS-cys that had counts of 30-300 colonies. The selected colonies were transferred to coded cryovials and stored at -20 °C (Microbank, Prolab Diagnostics, UK). As backup, in addition to randomly selecting individual colonies, a loop was used to harvest

all remaining colonies from the surface of the plate. The loopful was transferred to another coded cryovial. This provided a backup of additional microorganisms. The cryovials were stored in the freezer at -20 ^{o}C in Nigeria. They were transported to the UK in an insulated cooler bag. The total journey time was approximately nine hours.

Beads from single colonies and harvested colonies in the cryovials were cultured on MRS agar and incubated at 37 ^{o}C for 48-72 h in an anaerobic cabinet (Don Whitley, UK) in an atmosphere of 85% N, 10% H and 5% CO_2. Single colonies from both the random selection and the harvested colonies were streaked on MRS agar and incubated at 37 ^{o}C for 48-72 h in an anaerobic cabinet. Gram staining, catalase and oxidase tests were carried out on presumptive LAB. Single colonies from the plates were transferred aseptically into cryovials and stored at -20 ^{o}C until required for further tests.

2.2.5 Phenotypic Identification of Isolates

Pure cultures were streaked on MRS agar and incubated at 37 ^{o}C for 48-72 h in an anaerobic cabinet. Gram staining, catalase and oxidase tests were carried out.

2.2.5.1 Catalase and oxidase tests

For the catalase test, a drop of 30% (v/v) hydrogen peroxide (H1009 Sigma, Gillingham, UK), was placed on a single colony from a 24 h culture that had been transferred to a glass slide. Formation of bubbles of oxygen indicated a positive reaction. For the oxidase test, Whatman filter paper (10292221 Fischer Scientific, Leicestershire, UK) was saturated with oxidase reagent (Tetramethyl-p-phenylene

diamine dihydrochloride, 07770 Sigma, Gillingham, UK). A sterile plastic loop was used to pick a colony which was smeared on the saturated filter paper. A change in colour from colourless to blue within 20 s indicated a positive reaction due to formation of cytochrome c oxidase (Downes and Ito, 2001).

2.2.6 Genotypic assessment and identification of isolates

The methods of Ouoba *et al*. (2012) and Anyogu *et al*. (2014) were used for the genotypic evaluation of the isolates.

2.2.6.1 Chromosomal DNA extraction

A colony from a 24 h culture was inoculated in 1ml of autoclaved high purity water (W4502 Sigma, Gillingham, UK) in a 1.5ml Eppendorf centrifuge tube. The tube was centrifuged for 1 minute at 12,000 x g (Eppendorf 5415R, UK) to wash the cells. The supernatant was carefully discarded. An aliquot of 100 µl of InstaGene matrix (7326030 Bio-Rad, Hertfordshire, UK) was added to the pellet and incubated at 56 ^{o}C for 30 min. The tube was rotated on a vortex (Fisons, UK) for 10 s and placed in a 100 ^{o}C heat block for 8-10 min. The tube was again rotated on the vortex for another 10 s and centrifuged at 12,000 x g (Eppendorf 5415R, UK) for 3 min. The supernatant was transferred into a sterile Eppendorf tube. The DNA extract (supernatant) was stored at -20 ^{o}C for subsequent use.

2.2.6.2 Differentiation of isolates using rep-PCR

The rep-PCR molecular technique was used to group isolates that were isolated from human breast milk. Differentiation of isolates by rep-PCR was based on similarity of DNA band patterns. To conduct the PCR, a final volume of 25 µl

reaction mixture was set up as follows; 2 µl of extracted DNA, 2.5 µl of PCR buffer (10 X; N808-0161, Applied Biosystems), 4 µl of dNTP (1.25 mmol/ l; U1511,Promega, Southampton, UK), 2 µl of $MgCl_2$ (25 mmol/ l; AM9530G, Applied Biosystems), 4 µl of primer GTG5 (5'-GTG GTG GTGGTG GTG-3') (5 mmol/ l; Applied Biosystems), 0.2 µl of Taq polymerase (5 U; N808-0161, Applied Biosystems) and 10.30 µl of autoclaved high purity water (W4502 Sigma, Gillingham, UK). The amplification was carried out in a thermocycler (GeneAmp PCR 2700 system, Applied Biosystems, UK) using the following conditions; initial denaturation at 94 °C for 4 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 45 °C for 1 min, elongation at 65 °C for 8 min and final extension at 65 °C for 16 min, then cooling at 4 °C. The amplified fragments of DNA (PCR products) were separated using agarose gel 1.5 % (w/v) (1613103 Bio-Rad, Hertfordshire, UK) and electrophoresis at 120V for 3 h. PCR products were visualised after staining with diluted ethidium bromide (E1510 Sigma, Gillingham, UK) solution using a gel documentation system (GelDoc, UVP, Cambridge UK).

2.2.6.3 16S rRNA gene sequencing amplification

Representative isolates from each rep-PCR group were selected for16S rRNA gene amplification. Primers (100 mM) 0.5 µl of Primers (100 mM), PA (sequence) and 0.5 µl PE, (sequence) [Sigma, Gillingham, UK] were used to direct the amplification of a partial portion of the 16S rRNA gene. The reaction mixture consisted of 37.73 µl autoclaved high purity water (W4502 Sigma, Gillingham, UK), 5 µl 10x Buffer PCR buffer with $MgCl_2$ (Applied Biosystems,

UK), 5 µl dNTP 1.25 mM (Promega, UK), 0.25 µl Taq DNA Polymerase (Applied Biosystems, UK) and 1µl extracted DNA. A volume of 1µl of the extracted DNA was placed in a PCR tube and 49µl of the PCR mixture was added and mixed. The PCR tubes were placed in the DNA thermocycler (Applied Biosytems, UK). The amplification process included; denaturation at 95 ^{o}C for 5 min, 35cycles of 94 ^{o}C for 1 min, 55 ^{o}C for 1 min and 72 ^{o}C for 1min and a final extension at 72 ^{o}C for 5 min. In order to confirm that amplification had occurred, gel electrophoresis was carried out for 30 min as described in rep-PCR. PCR products that showed visible bands were purified using QIAquik PCR purification kit (Qiagen, UK), the manufacturer's instruction were followed. To generate a copy of the reverse strand, sequencing PCR was carried out using primer PD (sequence) (Sigma, Gillingham, UK) and the ABI big dye terminator (Applied Biosytems, UK). A volume of 4 µl of the purified PCR product was placed in a PCR tube, 2 µl of primer pD and 4 µl of ABI big dye terminator reaction mix were added and mixed thoroughly. The amplification process was performed under the following conditions; 95 ^{o}C for 2 min, then 35 cycles of 96 ^{o}C for 15 s, 40 ^{o}C for 1 s and 60 ^{o}C for 4 min. PCR products were sent to Source Bioscience (Cambridge, UK) for sequencing. Sequences of each isolate were compared to the GenBank database using the Basic Local Alignment Search Tool (BLAST) program of the National Center for Biotechnology Information (NCBI, MD, USA) and the EZTaxon (Yoon *et al.*, 2017) database. Genus and species identification of the isolates was confirmed when sequence showed above 97% (Genbank) and 97% (EZTaxon) similarity to those in the databases.

2.3 Results

2.3.1 Enumeration and isolation

In total, 20 volunteers were recruited in Nigeria following the procedures explained in section 2.2.1 (donor recruitment). Colonies were isolated from 18 of the 20 human breast milk samples examined which showed numbers ranging from 2.30±0.23 to 4.04±0.20 cfu/ml in MRS agar and 2.14±0.20 to 4.14±0.13 cfu/ml in MRScys agar. No growth was observed in two samples (samples B and N). No growth was also observed on MRScys agar that was inoculated with sample O. The numbers of colonies in the samples were similar in both the MRS and MRS-cys agar plates. Except for samples F and J where the number of colonies was higher in MRS (4.04±0.20) and in MRS-cys agar (4.14±0.13) respectively as compared to the rest of the samples (Table 2.1). A total of 108 isolates were isolated.

2.3.2 Phenotypic characteristics

One hundred and eight (108) isolates from MRS and MRS-cys agar were screened for their microscopic and biochemical characteristics (Table 2.2). All tested colonies from all the samples were Gram positive and oxidase negative. All isolates from samples A, C and F were catalase negative cocci, while all isolates from samples E, H and J were catalase positive cocci. All isolates from D and M were catalase negative rods. Isolates from sample G were catalase positive cocci, with the exception of isolates G1d and G1b (MRS) which were catalase negative. Sample I yielded mainly catalase positive cocci, but one was catalase negative. All isolates from Sample K contained principally catalase positive cocci except

for one, which was catalase negative. All isolates from Sample L were catalase positive; two of them (MRS) were rods and the other two cocci. Two isolates from sample O (MRS) were catalase positive rods, the other two were a rod and a coccus, both catalase negative. All the isolates from sample P were catalase negative, four were rods and one was a coccus. Sample Q yielded two catalase positive cocci and the other two were rods, one catalase negative and the other catalase positive. Three of the isolates from sample R (MRS) were catalase negative rods while the other three were catalase positive cocci. All isolates from sample S were catalase positive rods. One of the isolates from sample T (MRS) was catalase positive cocci; the others were catalase negative rods.

Table 2.1 The recovery of microorganisms from human breast milk on MRS and MRS-cys agar.

Samples	cfu/ml (MRS)	cfu/ml (MRS-cys)
A	3.34 ± 0.22	3.23 ± 0.14
B	<1x10	<1x10
C	3.17 ± 0.10	3.23 ± 0.14
D	3.36 ± 0.13	3.27 ± 0.20
E	3.17 ± 0.10	3.11 ± 0.17
F	4.04 ± 0.20	2.96 ± 0.35
G	2.86 ± 0.22	2.30 ± 0.15
H	2.30 ± 0.23	2.49 ± 0.15
I	3.90 ± 0.30	2.92 ± 0.20
J	2.65 ± 0.29	4.14 ± 0.13
K	2.77 ± 0.33	2.96 ± 0.29
L	2.65 ± 0.10	2.84 ± 0.14
M	2.70 ± 0.17	2.94 ± 0.17
N	<1x10	<1x10
O	2.47 ± 0.22	<1x10
P	3.25 ± 0.11	3.34 ± 0.16
Q	3.17 ± 0.10	3.20 ± 0.12
R	2.98 ± 0.48	2.14 ± 0.24
S	3.07 ± 0.10	3.27 ± 0.11
T	3.04 ± 0.21	2.93 ± 0.29

MRS = de Man Rogosa Sharpe agar

MRS-cys = de Man Rogosa Sharpe agar + L-cysteine

cfu/ml = colony forming unit per milliliter

Data are mean $\log_{10}$ ± standard deviation of duplicate experiments.

Table 2.2: Phenotypic characteristic of bacteria isolated from human breast milk.

Sample	Media	Gram stain and morphology	Catalase test	Oxidase test
A1a ,b, c, d and A2a, b, c and d	MRS-CYS	Gram +ve cocci in pairs, chains and clusters	-ve	-ve
B	MRS-CYS	ng	ng	ng
B	MRS	ng	ng	ng
C1a	MRS	Gram +ve cocci in pairs, chains and clusters	-ve	-ve
C2a, b, c and d	MRS-CYS	Gram +ve cocci in pairs, chains and clusters	-ve	-ve
D1	MRS-CYS	Gram +ve rods singly or in pairs	-ve	-ve
D2	MRS	Gram +ve rods singly or in pairs	-ve	-ve
E2a, b, c and d	MRS-CYS	Gram +ve cocci singly, in pairs and tetrads	+ve	-ve
F1 and F2	MRS	Gram +ve cocci in pairs and chains	-ve	-ve
F1 and F2	MRS-CYS	Gram +ve cocci in pairs and chains	-ve	-ve
G1a and c	MRS	Gram +ve cocci in pairs, tetrads and chains	+ve	-ve
G1b and d	MRS	Gram +ve cocci in pairs, tetrads and chains	-ve	-ve
G1a, b and c	MRS-CYS	Gram +ve cocci in pairs, tetrads and chains	+ve	-ve
G2a, b, c and d	MRS-CYS	Gram +ve cocci in pairs, tetrads and chains	+ve	-ve
G2a, b and c	MRS	Gram +ve cocci singly, in pairs, and chains	+ve	-ve

a, b, c and d = individual distinct colonies from plates that were cultured from cryovial beads.

ng = no growth.

MRS = de Man Rogosa Sharpe agar

MRS-cys = de Man Rogosa Sharpe agar + L-cysteine

Table 2.2 continued

Sample	Media	Gram stain and morphology	Catalase test	Oxidase test
H1 and H2	MRS	Gram +ve cocci in pairs, tetrads and chains	+ve	-ve
H1 and H2	MRS-CYS	Gram +ve cocci in pairs, tetrads and chains	+ve	-ve
I1	MRS	Gram +ve cocci in pairs, tetrads and chains	-ve	-ve
I1 and I2	MRS-CYS	Gram +ve cocci in pairs, tetrads and chains	+ve	-ve
I2	MRS	Gram +ve cocci in pairs, tetrads and chains	+ve	-ve
J2a and b	MRS	Gram +ve cocci in pairs, tetrads and chains	+ve	-ve
J2a, b, c and d	MRS-CYS	Gram +ve cocci in pairs, tetrads and chains	+ve	-ve
K1	MRS	Gram +ve cocci in pairs, tetrads and chains	-ve	-ve
K1 and K2	MRS-CYS	Gram +ve cocci in pairs, tetrads and chains	+ve	-ve
K2	MRS	Gram +ve cocci in pairs, tetrads and chains	+ve	-ve

a, b, c and d = individual distinct colonies from plates that were cultured from cryovial beads.

ng = no growth.

MRS = de Man Rogosa Sharpe agar

MRS-cys = de Man Rogosa Sharpe agar + L-cysteine

Table 2.2 continued

Sample	Media	Gram stain and morphology	Catalase test	Oxidase test
L1 and L2	MRS	Gram +ve rods singly or in pairs	-ve	-ve
L1 and L2	MRS-CYS	Gram +ve cocci pairs and chains	-ve	-ve
M1 and M2	MRS-CYS	Gram +ve rods singly or in pairs	-ve	-ve
M1, M2a ,b, c and M3	MRS	Gram +ve rods singly or in pairs	-ve	-ve
N	MRS	ng	ng	ng
N	MRS-CYS	ng	ng	ng
O	MRS-CYS	ng	ng	ng
O1	MRS	Gram +ve rods and cocci in pairs or chains	+ve	-ve
O2 and O3	MRS	Gram +ve rods singly or in pairs	-ve	-ve
P1	MRS	Gram +ve cocci in pairs, tetrads and chains	-ve	-ve
P2 and P3	MRS	Gram +ve rods singly or in pairs	-ve	-ve
P2a, b	MRS-CYS	Gram +ve rods singly or in pairs	-ve	-ve
Q1	MRS	Gram +ve cocci in pairs and chains	-ve	-ve
Q1	MRS-CYS	Gram +ve rods singly or in pairs	+ve	-ve

a, b, c and d = individual distinct colonies from plates that were cultured from cryovial beads.

ng = no growth.

MRS = de Man Rogosa Sharpe agar

MRS-cys = de Man Rogosa Sharpe agar + L-cysteine

Table 2.2 continued

Sample	Media	Gram stain and morphology	Catalase test	Oxidase test
Q2 and Q3a, b, c	MRS-CYS	Gram +ve rods singly or in pairs	-ve	-ve
Q2a	MRS	Gram +ve rods singly or in pairs	-ve	-ve
Q2b	MRS	Gram +ve cocci in pairs	+ve	-ve
R1	MRS	Gram +ve cocci in pairs, tetrads and chains	+ve	-ve
R1	MRS-CYS	Gram +ve cocci in pairs and chains	+ve	-ve
R2, R3 and R4a	MRS	Gram +ve cocci in pairs and chains	+ve	-ve
R4b, R5a and R5b	MRS	Gram +ve rods singly or in pairs	-ve	-ve
S1 and S2a, b	MRS-CYS	Gram +ve rods singly or in pairs	+ve	-ve
S3	MRS	Gram +ve rods singly or in pairs	+ve	-ve
T1, T2a, b and T3a, b	MRS-CYS	Gram +ve rods singly or in pairs	-ve	-ve
T1	MRS	Gram +ve cocci in pair and chains	+ve	-ve
T2a	MRS-CYS	Gram +ve rods singly or in pairs	-ve	-ve
T2b	MRS-CYS	Gram +ve rods singly or in pairs	-ve	-ve
T2a,b,c and T3a,b	MRS	Gram+ve rods singly or in pairs	-ve	-ve

a, b, c and d = individual distinct colonies from plates that were cultured from cryovial beads

ng = no growth.

MRS = de Man Rogosa Sharpe agar

MRS-cys = de Man Rogosa Sharpe agar + L-cysteine

2.3.2 Genotypic identification

The rep-PCR was first used to discriminate bacterial strains (Figure 2.1 to 2.5). This facilitated grouping of isolates into 19 groups based on visual observation of similarity of DNA band patterns. Using 16S rRNA gene sequencing, GenBank and EZTaxon databases, the isolates were identified. The identified organisms are shown in Table 2.3. Intraspecies genotypic diversity was seen in *Lactobacillus pentosus, Lactobacillus coryniformis* and *Leuconostoc mesenteroides* subsp. *lactis* which were divided into three and two groups respectively by rep-PCR (Table 2.3). Most of the identified LAB belong to the *Lactobacillus* genus, the rest are *Leuconostoc, Weissella, Streptococcus* and *Enterococcus*. The non-LAB were identified as *Staphylococcus epidermidis* and *Staphylococcus hominis.*

The predominant genus was *Lactobacillus* with four species; *Lactobacillus fermentum, Lactobacillus coryniformis, Lactobacillus pentosus* and *Lactobacillus plantarum. Weissella, Leuconostoc* and *Streptococcus* each have two species which are *Weissella confusa, Weissella paramesenteroides, Leuconostoc mesenteroides* subsp. *mesenteroides, Leuconostoc mesenteroides* subsp. *lactis, Streptococcus salivarius* subsp. *thermophilus* and *Streptococcus cristatus* respectively, and finally *Enterococcus faecium.* In total, 67% of the identified bacteria were LAB (*Enterococcus faecium* 8.8%, *Leuconostoc mesenteroides* subsp. *mesenteroides* 5.3%, *Leuconostoc mesenteroides* subsp. *lactis* 5.3%, *Lactobacillus fermentum* 5.3%, *Lactobacillus coryniformis* 5.4%, *Lactobacillus pentosus* 10.5%, *Lactobacillus plantarum* 1.8%, *Streptococcus salivarius* subsp.

thermophilus 3.5%, *Streptococcus cristatus* 1.8% *Weissella confusa* 10.5% and *Weissella paramesenteroides* 8.8%). The remaining 33% were non-LAB (*Staphylococcus epidermidis* 24.2% and *Staphylococcus hominis* 8.8%).

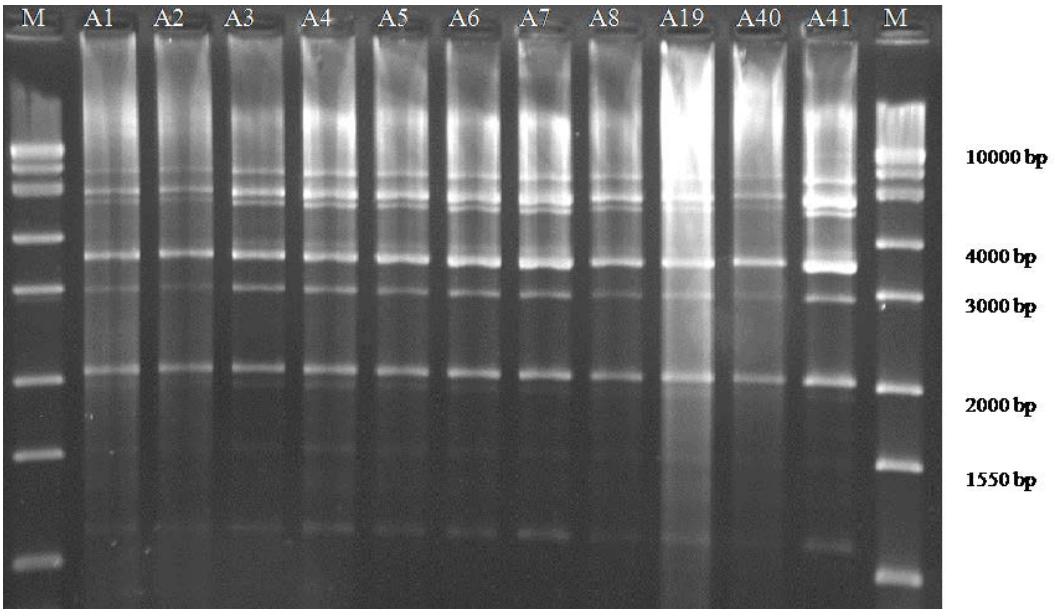

Figure 2.1: Rep-PCR gel image of DNA profiles of *Enterococcus faecium*

A1a[Mc] = A1, A1b[Mc] = A2, A1c=A3, A1d[Mc] = A4, A2a[Mc] = A5, A2b[Mc] = A6, A2c[Mc] = A7, A2d[Mc] =A8, C1a[M] = A19, G1b[M] = A40, G1d[M] = A41

M = Molecular marker

A1a [Mc] to G1d [M] = isolate codes

A1 to A41 = Rep-PCR profiles of isolates

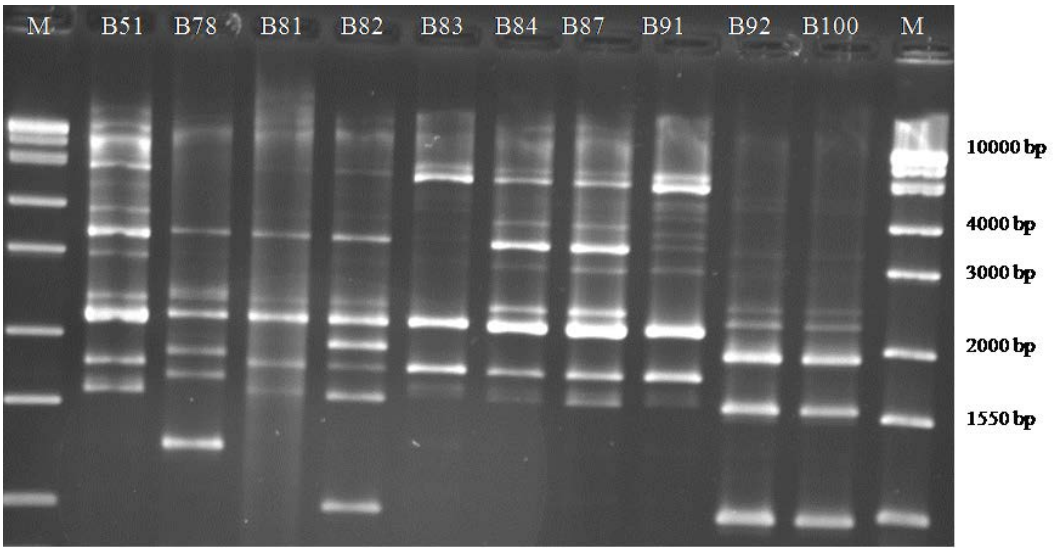

Figure 2.2: Rep-PCR gel image of DNA profiles of *Lactobacillus pentosus*

L1 [M] = B51, T1 [M] = B78, T2b [Mc] = B81, T2a [M] = B82, T2b [M] =B83,

T2c [M] = B84,

T3a [Mc] = B87, J2c [Mc] = B91, J2d [Mc] = B92, Q3b [Mc] = B100

M = Molecular marker

L1 [M] to Q3b [Mc] = isolate codes

B51 to B100 = Rep-PCR profiles of isolates

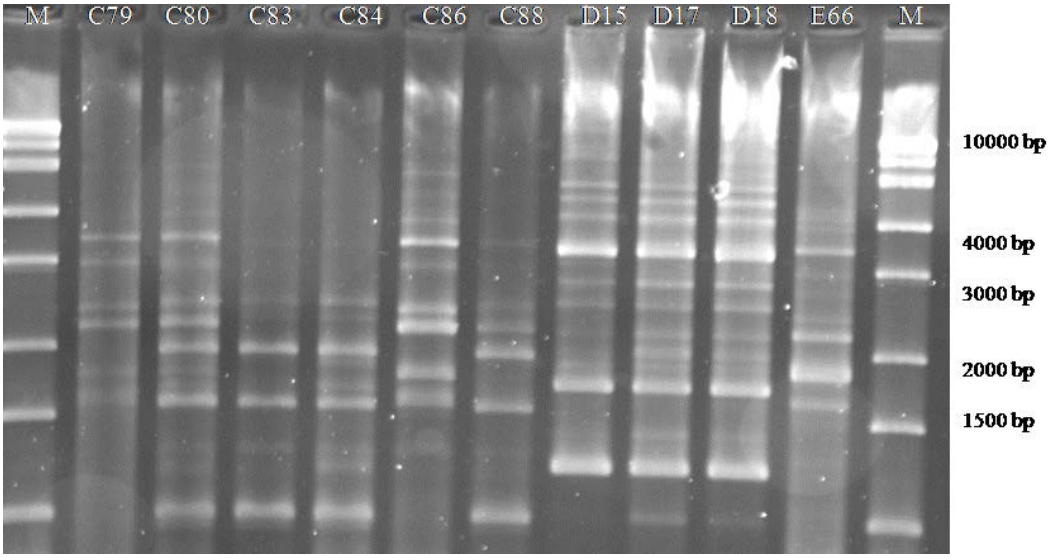

Figure 2.3: Rep-PCR gel image of DNA profiles of C = *Lactobacillus coryniformis*

T1 [Mc] = C79, T2a [Mc] = C80, T2b [M] = C83, T2c [M] = C84, T3b [Mc] = C86 and T3b [M] = C88

D = *Lactobacillus fermentum* E2d [Mc] = D15, O1 [M] = D17 and O2 [M] =D17

E = *Lactobacillus plantarum* P3 [M] = E66 and M = Molecular marker

T1 [Mc] to P3 [M] = isolate codes

C79 to E66 = Rep-PCR profiles of isolates

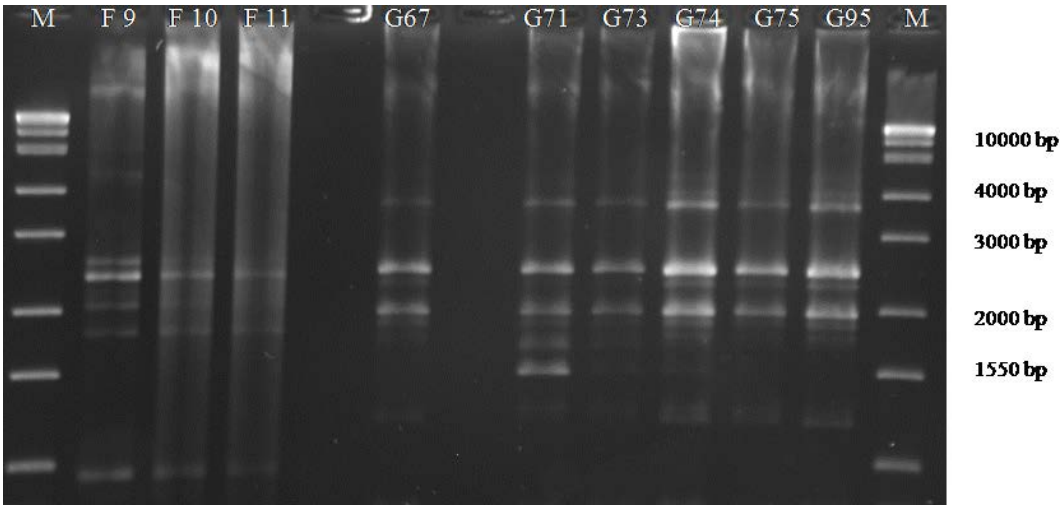

Figure2.4: Rep-PCR gel image of DNA profiles of F= *Leuconostoc mesenteroides* subsp. *mesenteroides*

C2a [M] = F9, C2b [Mc] = F10 and C2c [Mc] = F11

G = *Leuconostoc mesenteroides* subsp. *lactis*

R1 [M] = G67, J2b [M] = G71, Q3c [M] = G73, R4b [M] = G74, R5a [M] = 75,

Q1 [Mc] = G95

M = Molecular marker

C2a [M] to Q1 [Mc] = isolate codes

F9 to G95 = Rep-PCR profiles of isolates

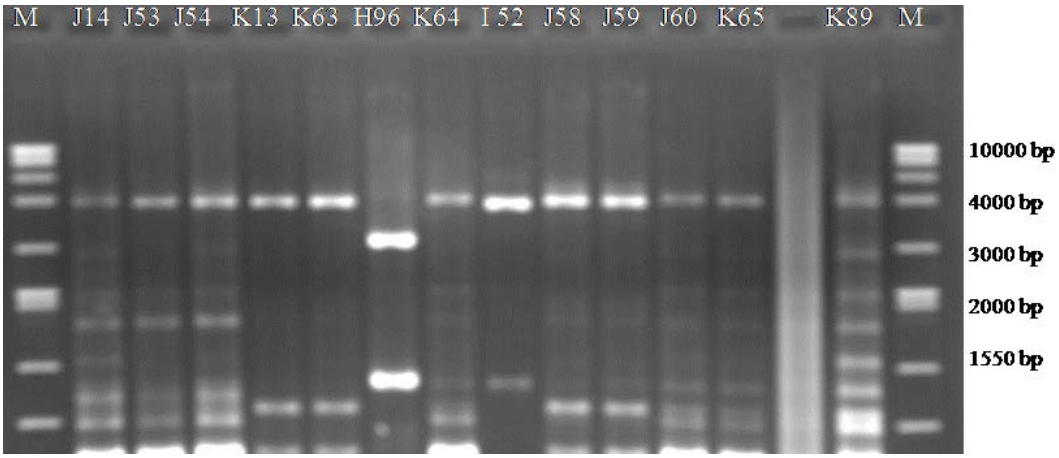

Figure 2.5: Rep-PCR gel image of DNA profiles of J = *Weissella confusa*

D2 [M] =J14, L2 [M] = J53, L2 [Mc] = J54, M2a [M] = J58, M2b [M] = J59 and M2c [M] = J60

H=*Streptococcus cristatus* Q1 [M] = H96,

I = *Streptococcus salivarius* subsp. *thermophilus* =L1 (Mc)

K = *Weissella paramesenteroides* D1 [Mc] = K13, P2 [M] = K63, P2a [Mc] = K64, P2b [Mc] = K65 and J2a [Mc] = K89

M = Molecular marker

D2 [M] to J2a (Mc) = isolate codes

J14 to K89 = Rep-PCR profiles of isolates

Table 2.3: Identification of 16S rRNA gene sequences of bacteria isolated from human breast milk by NCBI and EzTaxon databases.

Sample	Isolate code	Rep-PCR groups	Identification by 16S rRNA gene sequencing by NBCI BLAST	%	Confirmation of identification by Eztaxon	%
A	A1a (Mc)	1	*Enterococcus faecium*(B1)	98	*Enterococcus faecium*(B1)	99.39
	A1b (Mc)	1	*Enterococcus faecium*(B2)	98	*Enterococcus faecium*(B2)	98.86
B	ng	ng	ng	ng	ng	ng
C	C1a(M)	1	*Enterococcus faecium* (B19)	99	*Enterococcus faecium* (B19)	99.79
	C2b (Mc)	2	*Leuconostoc mesenteroides* subsp. *mesenteroides* (B10)	99	*Leuconostoc mesenteroides* subsp. *mesenteroides*(B10)	100
			Leuconostoc pseudomesenteroides	98		
	C2c (Mc)	2	*Leuconostoc mesenteroides* subsp. *mesenteroides*(B11)	99	*Leuconostoc mesenteroides* subsp. *mesenteroides*(B11)	100
	C2a (M)	2	*Leuconostoc Pseudomesenteroides*	98		
			Leuconostoc mesenteroides subsp. *mesenteroides(*B9)	99	*Leuconostoc mesenteroides* subsp. *mesenteroides(*B9)	100
D	D1 (Mc)	3	*Weissella paramesenteroides* (B13)	99	*Weissella paramesenteroides* (B13)	99.80
	D2 (M)	9	*Weissella confusa (B14)*	99	*Weissella confusa (B14)*	99.79
E	E2d (Mc)	4	*Lactobacillus fermentum* (B15)	100	*Lactobacillus fermentum* (B15)	99.17
	E2a (Mc)	5	*Staphylococcus epidermidis* (B20)	100	*Staphylococcus epidermidis* (B20)	100
	E2c (Mc)	5	*Staphylococcus epidermidis* (B22)	100	*Staphylococcus epidermidis* (B22)	99.12
F	F2 (M)	5	*Staphylococcus epidermidis (B32)*	96	*Staphylococcus epidermidis (B32)*	95.45

Mc = MRS-cysteine

M = MRS (B1) to (B88) = chromosal DNA codes

% = percent likelihood of identity (values in red = acceptable level of similarity)

ng = no growth

Table 2.3 continued

Sample	Isolate code	Rep-PCR groups	Identification by 16S rRNA gene sequencing by NBCI BLAST	%	Confirmation of identification by Eztaxon	%
G	G1b (M)	1	*Enterococcus faecium*(B40)	98	*Enterococcus faecium*(B40)	99.34
	G1d (M)	1	*Enterococcus faecium* (B41)	98	*Enterococcus faecium* (B41)	99.37
	G2a (M)	5	*Staphylococcus epidermidis* (B42)	99	*Staphylococcus epidermidis* (B42)	98.91
	G2b (M)	5	*Staphylococcus epidermidis* (B43)	98	*Staphylococcus epidermidis* (B43)	99.56
	G1a (Mc)	5	*Staphylococcus epidermidis (*B33) *Staphylococcus haemolyticus*	99 99	*Staphylococcus epidermidis (*B33)	97.99
	G2b (Mc)	5	*Staphylococcus epidermidis* (B36) *Staphylococcus haemolyticus*	99 99	*Staphylococcus epidermidis* (B36)	98.71
	G2c (Mc)	5	*Staphylococcus epidermidis (B38)* *Staphylococcus haemolyticus*	99 99	*Staphylococcus epidermidis (B38)*	98.73
	G1c (M)	6	*Staphylococcus hominis* (B47)	99	*Staphylococcus hominis* (B47)	100
	G2c (Mc)	6	*Staphylococcus hominis(*B37) *Staphylococcus warneri*	100 100	*Staphylococcus hominis(*B37)	99.79

Mc = MRS-cysteine

M = MRS (B1) to (B88) = chromosal DNA codes

% = percent likelihood of identity (values in red = acceptable level of similarity)

Table 2.3 continued

Sample	Isolate code	Rep-PCR groups	Identification by 16S rRNA gene sequencing by NBCI BLAST	%	Confirmation of identification by Eztaxon	%
H	H1 (Mc)	5	*Staphylococcus epidermidis* (B45)	100	*Staphylococcus epidermidis* (B45)	100
	H1 (Mc)	6	*Staphylococcus hominis* (B23)	100	*Staphylococcus hominis* (B23)	99.57
	H2 (Mc)	6	*Staphylococcus hominis* (B24)	100	*Staphylococcus hominis* (B24)	99.79
I	I2 (Mc)	18	*Pantoea dispersa (B28)*	93	*Pantoea dispersa (B28)*	89.84
J	J2a (Mc)	3	*Weissella paramesenteroides* (B89)	99	*Weissella paramesenteroides* (B89)	99.60
	J2d (Mc)	12	*Lactobacillus plantarum* (B92)	100	*Lactobacillus pentosus* (B92)	100
			Lactobacillus pentosus	99		
K	K1 (Mc)	5	*Staphylococcus epidermidis* (B48)	97	*Staphylococcus epidermidis* (B48)	97.33
	K1 (M)	5	*Staphylococcus epidermidis*(B94)	100	*Staphylococcus epidermidis* (B94)	99.79
L	L1 (M)	7	*Lactobacillus pentosus* (B51)	97	*Lactobacillus pentosus* (B51)	97.88
			Lactobacillus plantarum	97		
	L1 (Mc)	8	*Streptococcus salivarius* subsp. *thermophilus*(B52)	99	*Streptococcus salivarius* subsp. *thermophilus*(B52)	100
			Streptococcus sp. oral clone	99		
	L2 (Mc)	9	*Weissella confusa* (B54)	99	*Weissella confusa* (B54)	99.54
	L2 (M)	9	*Weissella confusa (B53)*	97	*Weissella confusa (B53)*	99.11

Mc = MRS-cysteine

M = MRS (B1) to (B88) = chromosal DNA codes

% = percent likelihood of identity (values in red = acceptable level of similarity)

Table 2.3 continued

Sample	Isolate code	Rep-PCR groups	Identification by 16S rRNA gene sequencing by NBCI BLAST	%	Confirmation of identification by Eztaxon	%
L	L1 (M)	7	*Lactobacillus pentosus* (B51)	97	*Lactobacillus pentosus* (B51)	97.88
			Lactobacillus plantarum	97		
	L1 (Mc)	8	*Streptococcus salivarius* subsp. *thermophilus* (B52)	99	*Streptococcus salivarius* subsp. *thermophilus* (B52)	100
			Streptococcus sp. oral clone	99		
	L2 (Mc)	9				
			Weissella confusa (B54)	99	*Weissella confusa* (B54)	99.54
	L2 (M)	9				
			Weissella confusa (B53)	97	*Weissella confusa (B53)*	99.11
M	M2b (M)	9	*Weissella confusa (B59)*	99	*Weissella confusa (B59)*	99.47
			Weissella koreensis	99		
			Weissella salipiscis	99		
	M2c (M)	9	*Weissella confusa (B60)*	100	*Weissella confusa (B60)*	99.77
	M2a (M)	9	*Weissella confusa* (B58)	90	*Weissella confusa* (B58)	99
N	ng	ng	ng	ng	ng	ng
O	O2 (M)	4	*Lactobacillus fermentum (B17)*	100	*Lactobacillus fermentum (B17)*	99.60
	O3 (M)	4	*Lactobacillus fermentum (B18)*	100	*Lactobacillus fermentum (B18)*	99.60

Mc = MRS-cysteine

M = MRS (B1) to (B88) = chromosal DNA codes

% = percent likelihood of identity (values in red = acceptable level of similarity)

ng = no growth

Table 2.3 continued

Sample	Isolate code	Rep-PCR groups	Identification by 16S rRNA gene sequencing by NBCI BLAST	%	Confirmation of identification by Eztaxon	%
P	P2a (Mc)	3	*Weissella paramesenteroides* (B64)	99	*Weissella paramesenteroides* (B64)	99.61
	P2b (Mc)	3	*Weissella paramesenteroides* (B65)	100	*Weissella paramesenteroides* (B65)	99.79
	P2 (M)	3	*Weissella paramesenteroides* (B63)	99	*Weissella paramesenteroides* (B63)	99.80
	P3 (M)	19	*Lactobacillus plantarum* (B66)	100	*Lactobacillus plantarum* (B66)	100
Q	Q2a (M)	6	*Staphylococcus hominis* (B98)	98	*Staphylococcus hominis* (B98)	98.38
	Q3c (M)	10	*Leuconostoc mesenteroides* subsp. *lactis*(B73)	99	*Leuconostoc mesenteroides* subsp. *lactis*(B73)	99.34
	Q1(Mc)	10	*Leuconostoc mesenteroides* subsp. *lactis*(B95)	99	*Leuconostoc mesenteroides* subsp. *lactis*(B95)	99.52
			Leuconostoc garlicum	99		
	Q2b (M)	11	*Streptococcus pneumoniae* (B72)	93	*Streptococcus pneumoniae* (B72)	82.42
			Streptococcus mitis	94		
	Q1 (M)	16	*Streptococcus cristatus* (B96)	100	*Streptococcus cristatus* (B96)	99
R	R1 (Mc)	5	*Staphylococcus epidermidis B(101)*	97	*Staphylococcus epidermidis B(101)*	100
	R1(M)	17	*Leuconostoc lactis(B67)*	99	*Leuconostoc lactis(B67)*	100
			Leuconostoc garlicum	99		
			Leuconostoc citreum	98		

Mc = MRS-cysteine

M = MRS (B1) to (B88) = chromosal DNA codes

% = percent likelihood of identity (values in red = acceptable level of similarity)

Table 2.3 continued

Sample	Isolate code	Rep-PCR groups	Identification by 16S rRNA gene sequencing by NBCI BLAST	%	Confirmation of identification by Eztaxon	%
S	S3 (M)	5	*Staphylococcus epidermidis* (B77)	99	*Staphylococcus epidermidis* (B77)	100
	S1 (Mc)	5	*Staphylococcus epidermidis* (B103)	100	*Staphylococcus epidermidis* (B103)	100
	S2b (Mc)	8	*Streptococcus salivarius* subsp. *thermophilus* (B76)	99	*Streptococcus salivarius* subsp. *thermophilus* (B76)	100
T	T1 (M)	12	*Lactobacillus pentosus* (B78)	96	*Lactobacillus pentosus* (B78)	100
			Lactobacillus plantarum	96		
	T2b (Mc)	12	*Lactobacillus pentosus* (B81)	100	*Lactobacillus pentosus* (B81)	98.04
			Lactobacillus plantarum	100		
	T2a (M)	12	*Lactobacillus pentosus* (B82)	97	*Lactobacillus pentosus* (B82)	100
			Lactobacillus plantarum	97		
	T3b (Mc)	13	*Lactobacillus coryniformis* (B86)	99	*Lactobacillus coryniformis* (B86)	99.79
	T2a (Mc)	13	*Lactobacillus coryniformis(B80)*	99	*Lactobacillus coryniformis(B80)*	99.58
			Lactobacillus salivarus	99		
			Lactobacillus rhamnosus	99		
	T3a (M)	14	*Lactobacillus pentosus* (B87)	100	*Lactobacillus pentosus* (B87)	99.56
			Lactobacillus plantarum	100		
	T3b (M)	15	*Lactobacillus coryniformis (*B88)	97	*Lactobacillus coryniformis (*B88)	99.79
			Lactobacillus rhamnosus	97		

Mc = MRS-cysteine

M = MRS (B1) to (B88) = chromosal DNA codes

% = percent likelihood of identity (values in red = acceptable level of similarity)

2.4 Discussion

It was imperative to investigate presence of LAB in human breast milk samples from Nigerian breast feeding mothers. Limited research on LAB in human breast milk exist in the African continent especially Nigeria. The presence of LAB in human breast milk was therefore investigated in this study. The laboratory analysis of the breast milk samples involved enumeration and isolation of LAB from breast milk samples and preliminary phenotypic identification of isolates including Gram staining, catalase and oxidase tests.

A total of 108 isolates from 20 samples of breast milk were recovered from both MRS agar and MRS-cys agar. The isolation of presumptive LAB from breast milk samples using MRS and MRS-cys agar is a good indication that potentially beneficial bacteria may be present in the milk samples. MRS-cys is MRS agar that has been supplemented with L-cysteine hydrochloride. L-cysteine hydrochloride facilitates removal of oxygen from MRS medium, creating more anaerobic condition which promotes growth of LAB and bifidobacteria.

Tusar *et al*. (2014) isolated 86 isolates from human breast milk of 11 Slovenian lactating mothers using MRS agar, M17 agar and Transgalctosylated oligiosaccharide (TOS) propionate agar. However, Nasiraii *et al*. (2011) isolated 306 isolates from human breast milk samples of 20 Iranian breast feeding mothers using MRS agar.

The isolation of 10^2 to 10^4 cfu/ml bacterial cells in this study corroborates with the counts of Martin *et al*. (2009) and Albesharat *et al*. (2011). Other research has shown that approximately 10^3 to 10^4 cfu/ml LAB have been isolated from the breast milk of healthy mothers (Jeurink *et al*. 2013). The standard minimum consumption of potentially beneficial bacterial cells per day for any value to the consumer is 10^6 to10^9cfu/ml or cfu/gram (Lourens-Hattingh and Viljoen, 2001 and WGO, 2008). The isolation of 10^2 to 10^4cfu/ml bacterial cells in this study indicates that the babies would ingest the required daily number of cells, since they will drink at least 700ml of breast milk per day. Infants therefore ingest $1x10^5$ to $1x10^7$ beneficial bacterial cells during suckling, if the babies consume approximately 800ml (Singh *et al*. 2014).

The genotyping of LAB is currently the most reliable method of verifying their identity at the genus, species and subspecies level. Using rep-PCR allowed typing of the isolates at genus, species and sub-species level into 19 groups based on their DNA patterns. The rep-PCR showed that there is an intraspecies genotypic diversity in some of the LAB. This was seen in *Lactobacillus pentosus*, *Lactobacillus coryniformis* and *Leuconostoc mesenteroides* subsp. *lactis.* No genotypic diversity was observed in *Enterococcus faecium, Leuconnostoc mesenteroides* subsp.*mesenteroides, Lactobacillus fermentum, Streptococcus cristatus*, *Streptococcus salivarius* subsp. *thermophilus*, *Weissella confusa* and *Weissella paramesenteroides*.

Both phenotypic and genotypic techniques were used in the identification of LAB in this study. Similar methods were also used by other researchers for

identification of LAB in human breast milk (Martin *et al*. 2003, Albesharat *et al*. 2011, Singh *et al*. 2014 and Tusar *et al*. 2014), human feces (Khalil *et al*. 2007 and Albesharat *et al*. 2011) and fermented foods (Kpikpi *et al*. 2010, Takeda *et al*. 2011, Ouoba *et al*. 2012 and Anyogu *et al*. 2014).

Research on human breast milk indicates similar LAB to those herein reported, and also other LAB and non-LAB not identified in this research. Martin *et al*. (2003) identified *Lactobacillus fermentum, Enterococcus faecium* and *Lactobacillus gasseri.* Albesharat *et al*. (2011) reported the presence of *Lactobacillus plantarum, Enterococcus faecium, Enterococcus faecalis, Lactobacillus fermentum, Lactobacillus brevis* and *Pediococcus pentosaceus,* while Tusar *et al*. (2014) identified *Lactobacillus fermentum, Enterococcus faecalis, Lactobacillus salivarius, Lactobacillus gasseri, Lactobacillus reuteri, Bifidobacterium breve* and *Staphylococcus epidermidis.* Maternal diet could account for some differences in LAB observed in this study to those reported by other authors such as Albesharat *et al*. (2011) and Tusar *et al*. (2014).

Publications reporting on fermented foods identified similar LAB to those isolated from human breast milk. Anyogu *et al*. (2014) identified *Lactobacillus plantarum, Enterococcus faecium, Leuconostoc mesenteroides* subsps.*mesenteroides, Weissella confusa* and *Weissella paramesenteroides* from fermented cassava, while Kpikpi *et al*. (2010) isolated *Lactobacillus plantarum, Lactobacillus fermentum* and *Leuconostoc mesenteroides* subsp. *mesenteroides* from fermented condiments. Takeda *et al*. (2011) recovered *Lactobacillus fermentum, Enterococcus faecium, Leuconostoc mesenteroides* subsp. *mesenteroides,*

Weissella confusa, *Lactobacillus plantarum* and *Lactobacillus pentosus* from fermented dairy products. It is interesting to note that seven out of the eleven identified LAB in this study are similar to LAB identified from fermented foods. Some studies have linked presence of same species of probiotic LAB ingested by mothers to those found in their breast milk (Nasiraii *et al*. 2011and Gomez-Gallago *et al*. 2016). Therefore, maternal dietary habits could contribute to diversity of LAB in human breast milk. Moreover, the origin of LAB present in human breast milk has been attributed to maternal gut (Martin *et al*. 2003 and Jeurink *et al*. 2013). Some bacteria could migrate from maternal gut to mammary gland with the help of maternal dendritic cells (Fernandenz *et al*. 2013).

The identification of LAB, *Staphylococcus epidermidis* and *Staphylococcus hominis* in this study is corroborated by the results of other studies on human breast milk such as Martin *et al*. (2003), Martin *et al*. (2007), Makino *et al*. (2011), Martin *et al*. (2012), Fernandez *et al*. (2013), Tusar *et al*. (2014) and Altuntas, (2015). In fact, Martin *et al*. (2012) stated that *Staphylococcus epidermidis* and *Staphylococcus hominis* were identified in the entire human breast milk samples they evaluated using 16S rRNA gene sequencing.

Martin *et al*. 2012 collected samples of breast milk from Spanish breast feeding mothers after cleansing of maternal nipples. But they reported the presence of *Staphylococcus epidermidis* and *Staphylococcus hominis* in the samples. Similarly, Tusar *et al*. 2014 used aseptic techniques for collection of breast milk samples from Slovenian lactating mothers. *Staphylococcus epidermidis* were also identified in the milk samples. However, Sakwanski *et al*. 2016 compared the

microbiota of Chinese human breast milk collected with aseptic cleansing and without aseptic cleansing of maternal nipples. *Staphylococcus* species were identified in both samples. Aseptic cleansing of nipples therefore, does not prevent the identification of *Staphylococcus* species from human breast milk. Moreover, *Staphylococcus* species are commensal bacteria inherent in human breast milk.

Some of the LAB identified from the Nigerian human breast milk were similar to those isolated in other countries, since *Enterococcus faecium* and *Lactobacillus* spp. were also identified in Spanish human breast milk (Martin *et al*. 2003), Slovenian human breast milk (Tusar *et al*. 2014) and from the breast milk of Syrian mothers (Albesharat *et al*. 2011).

LAB are important beneficial bacteria that have been used for centuries as starter organisms in the production of many kinds of fermented foods (Lee *et al*. 2012). Because of their ancient use in food production, some genera of LAB such as *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Leuconostoc* and *Pediococcus* have been "generally recognized as safe" (GRAS) by the United States Food and Drug Administration (USFDA) and have also been included in the list of "Qualified Presumption of Safety" (QPS) by the European Food Safety Authority (EFSA) (Ayeni *et al*. 2011).

Olivares *et al*. (2006) suggested that breast milk of healthy breast-feeding mothers is the best source of beneficial LAB for the infant gastrointestinal tracts (GIT). Breast fed babies have a more diverse population of LAB in their GITs than their formula-fed counterparts (Matsuki *et al*. 1999 and Gueimonde *et al*. 2007).

Human breast milk is therefore an important source of potentially beneficial LAB that has been shown to promote the health and well-being of newly born babies and infants.

2.5 Conclusion

Bacteria recovered from human breast milk samples belong to a diverse range of genera and species of LAB. The 16S rRNA gene sequencing identified 38 LAB, *Lactobacillus* was the predominant genus with four species which are; *Lactobacillus fermentum, Lactobacillus coryniformis, Lactobacillus pentosus* and *Lactobacillus plantarum.* This study has contributed to the knowledge of the diversity of LAB in some samples of Nigerian human breast milk. The identification of these bacteria has facilitated further research into their probiotic potential and antimicrobial resistance profile.

CHAPTER THREE: ACID AND BILE RESISTANCE STUDY OF LACTIC ACID BACTERIA

3.1 Introduction

For beneficial bacteria to improve the health and wellbeing of a host, it must resist the hostile environmental conditions of the upper gastrointestinal tract (GIT). The digestive system has various lethal, but host-protective, mechanisms that prevent passage of microorganisms through the GIT such as hydrochloric acid, gastric secretions and bile salts (Jin *et al*. 1998). As soon as LAB enter the duodenum, their survival depends on their ability to resist and adapt to bile salts, because the bacterial cell membrane consists of lipid and fatty acids that are prone to destruction by bile salts (Meritt and Donaldson, 2009).

The GIT is a systemic tube divided into segments from the mouth to the anus (Figure 3.1). The regions of the digestive system are: mouth, oesophagus stomach, small intestine and large intestine. The stomach and upper small intestine contain fewer bacteria (10^3 to 10^5 per gram of intestinal content) due to the low pH (2-4) of the segment. Acid tolerant *Lactobacillus* and *Streptococcus* are the dominant genera in this segment. The lower part of the small intestine (ileum) contains diverse bacterial communities (10^8 per gram of intestinal content), while the large intestine (colon) has pH value of 5.5 to 7.0. The large intestine is the most important location for bacterial colonization and is the most diverse and densely populated region (10^{11} to 10^{13} per gram of intestinal content (Mackie *et al*. 1999). The GIT of an unborn baby is sterile (Neu and Li, 2003), colonization of the GIT begins after birth with transmission of microbes from the mother and the immediate environment. Babies are further exposed to a range of bacteria by ingesting breast milk and other food substances. Bacteria entering the

GIT through breast feeding are more likely to colonise the infant gut than bacteria ingested in other foods (Albesharat *et al*. 2011) and factors that regulate the fate of ingested microorganisms differ between newborns and adults (Mackie *et al*. 1999). The stomach of the adult is more acidic than that of the newborn baby, the pH of the adult stomach is between 2 and 4. But in the first 24 to 48 hours after birth, the gastric pH of a baby is 5.5 to 7 (Neu and Li, 2003). The average time that a probiotic can survive in the stomach is 90 minutes (Park and Lim, 2015).

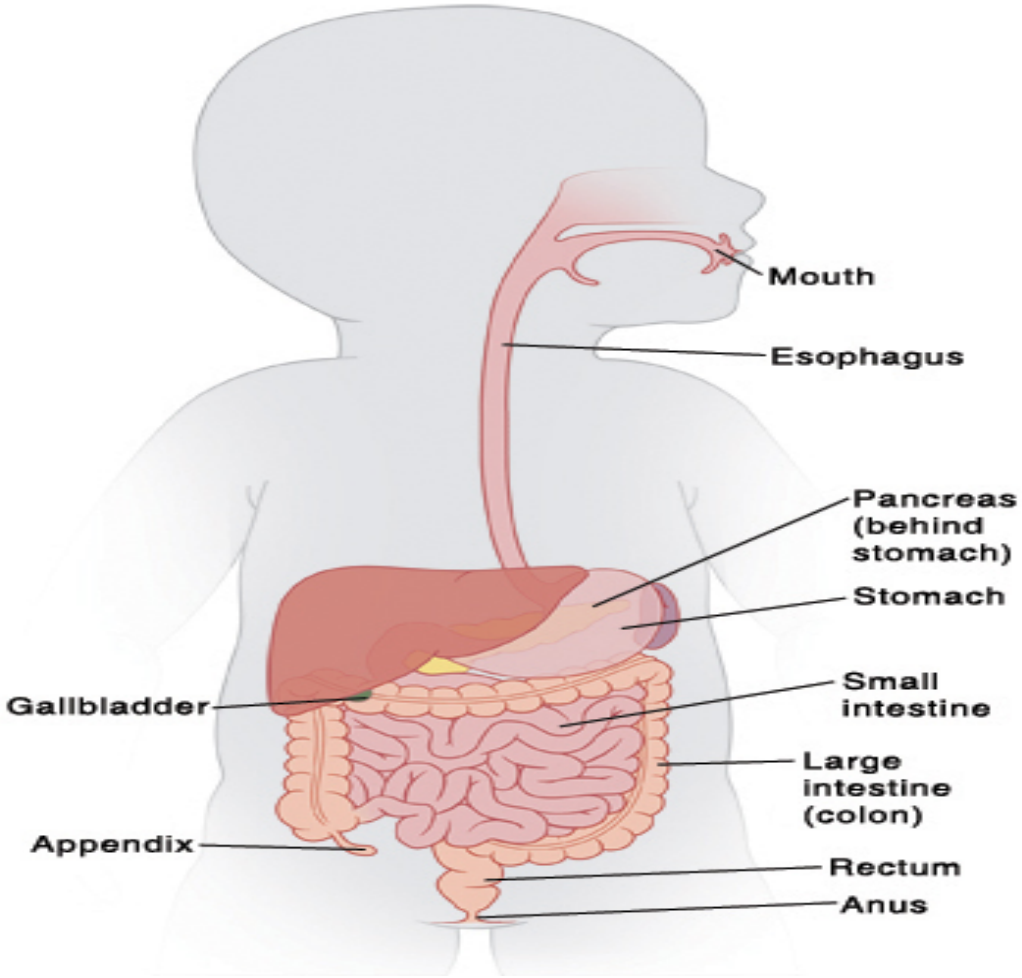

Figure 3.1: Anatomy of the pediatric digestive system. Source: Fairview Health Library (2016).

In vitro studies of acid and bile resistance are vital in assessing the capability of beneficial bacteria to survive in the GIT ((Pan *et al*. 2009, Bendali *et al*. 2011, Zhang *et al*. 2013and Park and Lim, 2015). Survival of LAB at low pH such as 2 to 4 is variable and dependent on the intrinsic (genotypic) attributes of a particular species, but supplementing acidic environments with glucose, lysozyme and pepsin increases the likelihood of survival of probiotic LAB (Corcoran *et al*. 2005, Hosseini *et al*. 2009, Bendali *et al*. 2011 and Zhang *et al*. 2013).

Resistance to bile toxicity is another important attribute of probiotic bacteria. Bile tolerance is species-specific and therefore tolerance amongst species is variable (Begley *et al*. 2005). Variability in resistance to bile salts within genera and species of LAB has been reported by Chateau *et al*. (1994) who recorded a diverse resistance pattern in strains of *Lactobacillus rhamnosus.* Jacobs *et al*. (1999) observed similar variability in strains of *Lactobacillus plantarum, Lactobacillus fermentum, Lactobacillus rhamnosus* and *Lactobacillus casei*.

Resistance of LAB to acid and bile is variable among genera and species of LAB, it is therefore necessary to screen some of the identified LAB in this study. The aim of the study described in this chapter was to examine tolerance of some of the identified LAB to acid and bile solutions *in vitro*. Ability of LAB to survive in simulated gastric pH and bile could attribute LAB to potential probiotic. Thus, enable further probiotic characterisation of selected LAB. The specific objectives of this study were:

- To assess the survivability of selected LAB in different simulated gastric pH values, with and without pepsin.
- To assess the survivability of selected LAB in different concentrations of bile salt.

The LAB selected for investigation were:

- *Enterococcus faecium* (A1a[Mc])
- *Lactobacillus coryniformis* (T3b[Mc])
- *Lactobacillus fermentum* (O3[M])
- *Lactobacillus pentosus* (T2a[M])
- *Lactobacillus plantarum* (P3[M])
- *Leuconostoc mesenteroides* subsp. *lactis* (R1[M])
- *Leuconostoc mesenteroides* subsp. *mesenteroides* (C2c[Mc])
- *Streptococcus cristatus* (Q1[M])
- *Streptococcus salivarius* subsp. *thermophilus* (S2b[Mc])
- *Weissella confusa* (M2c[M])
- *Weissella paramesenteroides* (D1[Mc])

Each of the LAB selected represent other identified LAB of the same genus and species. The selection was based on similarity in their rep-PCR profiles.

3.2 Materials and methods

The acid and bile resistance study was carried out according to the methods of Jin *et al*. (1998), Chung *et al*. (1999), Lee *et al*. (2012) and Singh *et al*. (2014).

3.2.1 Acid resistance study

Acid resistance of selected LAB was studied in phosphate buffered saline (PBS) with pH 7.3 as control, and PBS with pH adjusted to values of 4.0, 3.0 and 2.0 using 1M hydrochloric acid (HCl) without pepsin, and at the same pH values with pepsin (3g/L). The solutions were sterilised using 0.2 micron membrane filters (7187, Whatman, UK) under vacuum (model 2552C-02 Welch Thomas Stokie, USA), then aseptically transferred in 9ml volumes to sterile universal bottles. A loopful of several colonies of a 24 h culture of the isolate grown on MRS agar was sub-cultured into 1 ml maximum recovery diluent (MRD) and drops of this were added to 9 ml of MRD in sterile bottles with black caps until a value of 0.5 on the MacFarland standard was achieved, which gave an approximate concentration of 10^8 cfu/ml (Dalynn biological 2014), which constituted the inoculum for the experiment. One ml of the inoculum was placed in the control tube containing 9 ml PBS (pH 7.3), and the same volume was added to 9 ml tubes of adjusted PBS.The tubes were incubated anaerobically at 37 ^{o}C for 3 h during which 100 µl were withdrawn using a pipette every 30 m, decimally diluted and plated on MRS agar, which was incubated anaerobically at 37 ^{o}C for 48 h. The enumerations were carried out in duplicate, colonies counted and mean values

calculated as cfu/ml. Survival ratios were calculated by dividing the stressed cell number by the control cell number and multiplying by 100.

3.2.2 Bile resistance study

Bile resistance of the selected LAB were also studied in PBS (control) and PBS containing 0.3, 0.5, 1 and 1.5 % (w/v) bile salt (B3883, Sigma, UK). The solutions were filter sterilised and aseptically dispensed after sterilisation, as described in Section 3.1.1. The inoculum of LAB was prepared as described in Section 3.1.1 and inoculation of the tubes containing PBS with bile salts was also carried out in the same way. The tubes were incubated anaerobically at 37 ^{o}C for 3 h and samples taken every 30 min and treated as described in Section 3.1.1. The enumerations were carried out in duplicate, colonies counted and mean values calculated as cfu/ml. Survival ratios were calculated by dividing the stressed cell number by the control cell number and multiplying by 100.

3.3 Results

3.3.1 Acid and bile resistance study

Three different pH and bile concentrations were chosen to simulate the stomach acid and duodenum bile. The acid resistance of the eleven selected LAB were examined in PBS with pH adjusted to values of 4.0, 3.0 and 2.0 using 1M hydrochloric acid (HCl) without pepsin, and at the same pH values with pepsin (3g/L). The bile resistance was assessed in PBS containing 0.3, 0.5, 1 and 1.5 % (w/v) bile salt. The results of the acid and bile resistance of the studied lactic acid

bacteria are shown in figures 3.2 to 3.23 (graphs are $\log_{10}$ cfu/ml of bacterial counts, Tables are shown in Appendix 4).

Cells of *Leuconostoc mesenteroides* subsp. *mesenteroides* were completely killed when exposed to pH 2.0 + pepsin and pH 2.0 at 0 minutes. *Leuconostoc mesenteroides* subsps. *mesenteroides* only survived in pH 3.0 during the first 30 minutes, it did not survive beyond 30 minutes. In pH 3.0 + pepsin, cells survived the 3 hours of incubation. Pepsin therefore had a major effect on tolerance of *Leuconostoc mesenteroides* subsps. *mesenteroides* to pH 3 (Figure 3.2). Similarly, the bacterium was able to survive in pH 4.0 + pepsin and pH 4.0 without pepsin. Cells of *Leuconostoc mesenteroides* subsp. *mesenteroides* survived in all the bile concentrations of 0.3, 0.5, 1.0 and 1.5% up to the third hour (Figure 3.3).

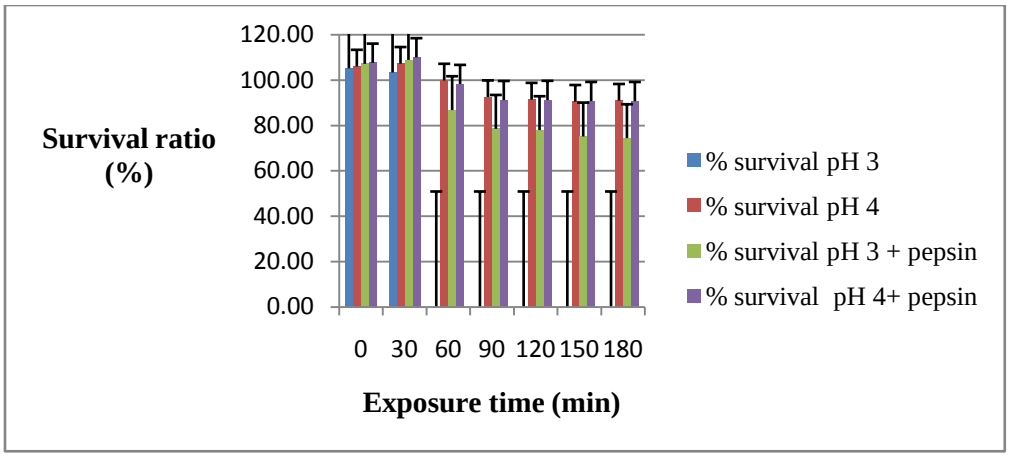

Figure 3.2: Percentage survival ratios of *Leuconostoc mesenteroides* subsp. *mesenteroides* at pH values of 3.0 and 4.0 with and without pepsin. The error bars are standard deviation of duplicate experiments.

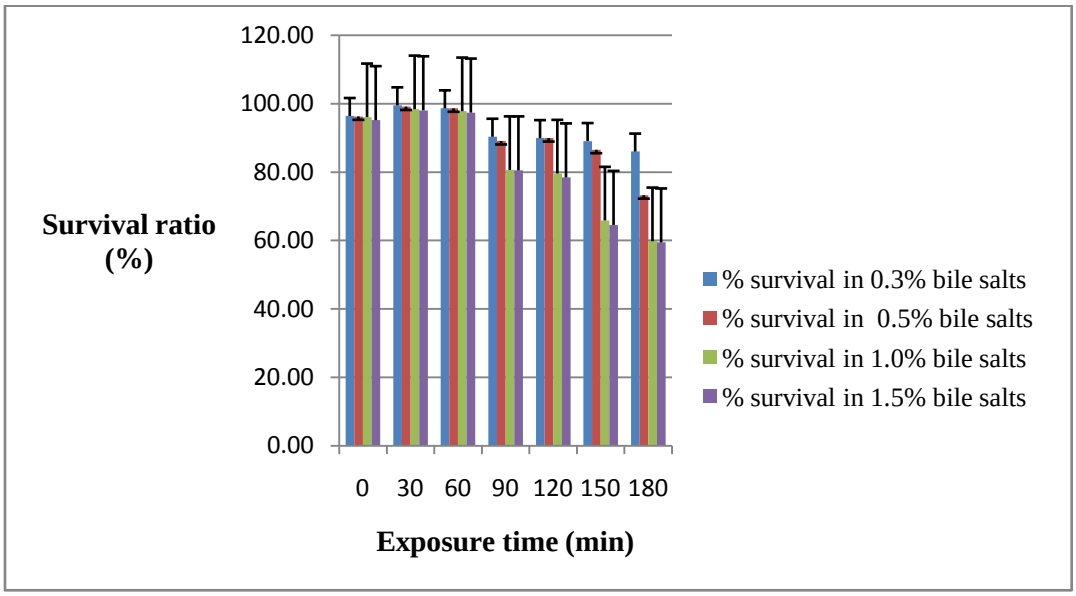

Figure 3.3: Percentage survival ratios of *Leuconostoc mesenteroides* subsp. *mesenteroides* in 0.3, 0.5, 1.0 and 1.5 % bile salts. The error bars are standard deviation of duplicate experiments.

Similarly, *Weissella paramesenteroides* could not survive in pH 2.0 + pepsin and pH 2.0 without pepsin. The cells did not survive beyond 30 minutes in pH 3.0

without pepsin. In pH 3.0 + pepsin, the cells survived up to the third hour indicating major effect of pepsin. In pH 4.0 + pepsin and pH 4.0 without pepsin, the cells also survived up to the third hour (Figure 3.4). Cells of *Weissella paramesenteroides* were able to survive in all the bile concentrations of 0.3, 0.5, 1.0 and 1.5 (Figure 3.5).

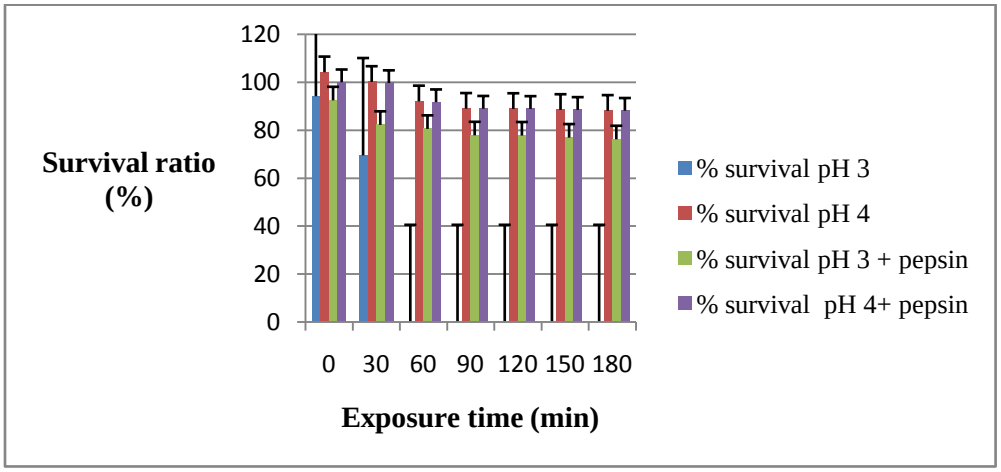

Figure 3.4: Percentage survival ratios of *Weisella paramesenteroides* at pH values of 3.0 and 4.0 with and without pepsin. The error bars are standard deviation of duplicate experiments.

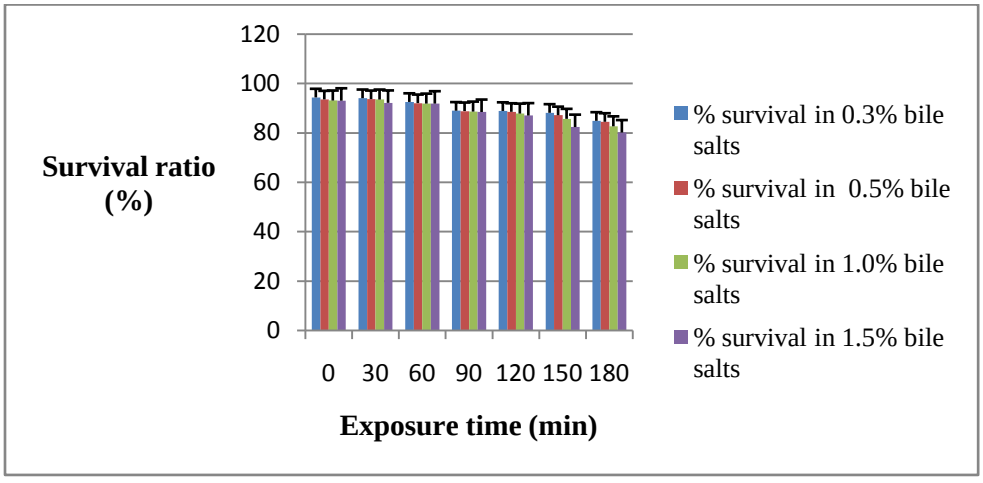

Figure 3.5: Percentage survival ratios of *Weisella paramesenteroides* in 0.3, 0.5, 1.0 and 1.5 % bile salts. The error bars are standard deviation of duplicate experiments.

Cells of *Lactobacillus fermentum* continued to survive in pH 2.0 + pepsin up to the third hour but the cells did not survive beyond the first hour in pH 2.0. In pH 3.0 + pepsin and pH 3.0 without pepsin, as well as pH 4.0 + pepsin and pH 4.0 without pepsin the cells survived up to the third hour (Figure 3.6). Resistance to bile salt was variable with time and concentration. Cells of *Lactobacillus fermentum* were able to survive up to three hours in 0.3% and 0.5%. The cells were able to survive up to two hours in 1.0 and 1.5% after which they became less tolerable (Figure 3.7).

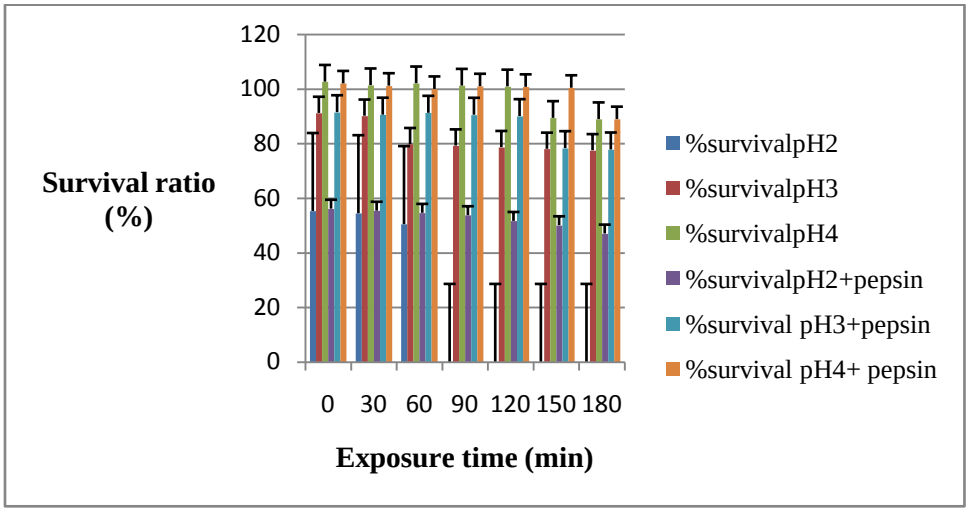

Figure 3.6: Percentage survival ratios of *Lactobacillus fermentum* at pH values of 2.0, 3.0 and 4.0 with and without pepsin. The error bars are standard deviation of duplicate experiments.

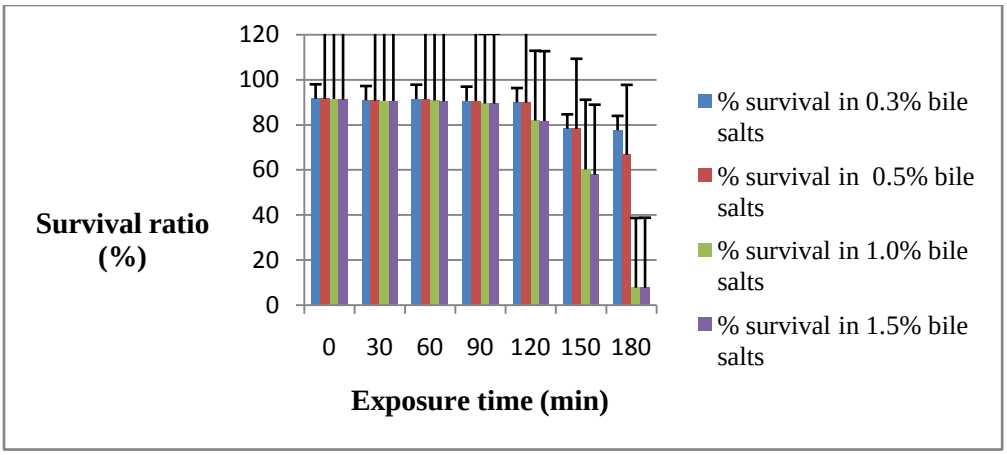

Figure 3.7: Percentage survival ratios of *Lactobacillus fermentum* in 0.3, 0.5, 1.0 and 1.5 % bile salts. The error bars are standard deviation of duplicate experiments.

Lactobacillus pentosus was able to survive in pH 2.0 + pepsin up to the three hours of incubation no survival was seen in pH 2.0 beyond zero minute. Pepsin therefore, had significant effect on survival of the cells at this pH.

In pH 3.0 + pepsin, pH 3.0 without pepsin, pH 4.0 + pepsin and pH 4.0 without pepsin the cells survived the three hours of incubation (Figure 3.8). Cells of *Lactobacillus pentosus* also survived in all the bile concentrations of 0.3, 0.5, 1 and 1.5% up to the third hour of incubation (Figure 3.9).

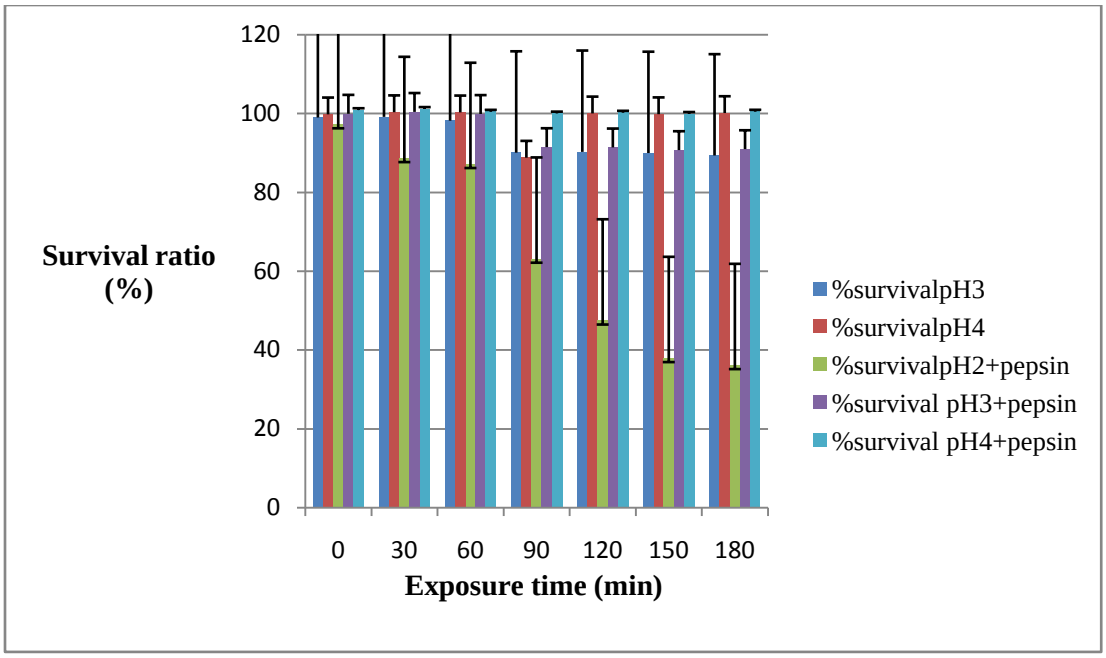

Figure 3.8: Percentage survival ratios of *Lactobacillus pentosus* at pH values of 2.0, 3.0 and 4.0 with and without pepsin. The error bars are standard deviation of duplicate experiments.

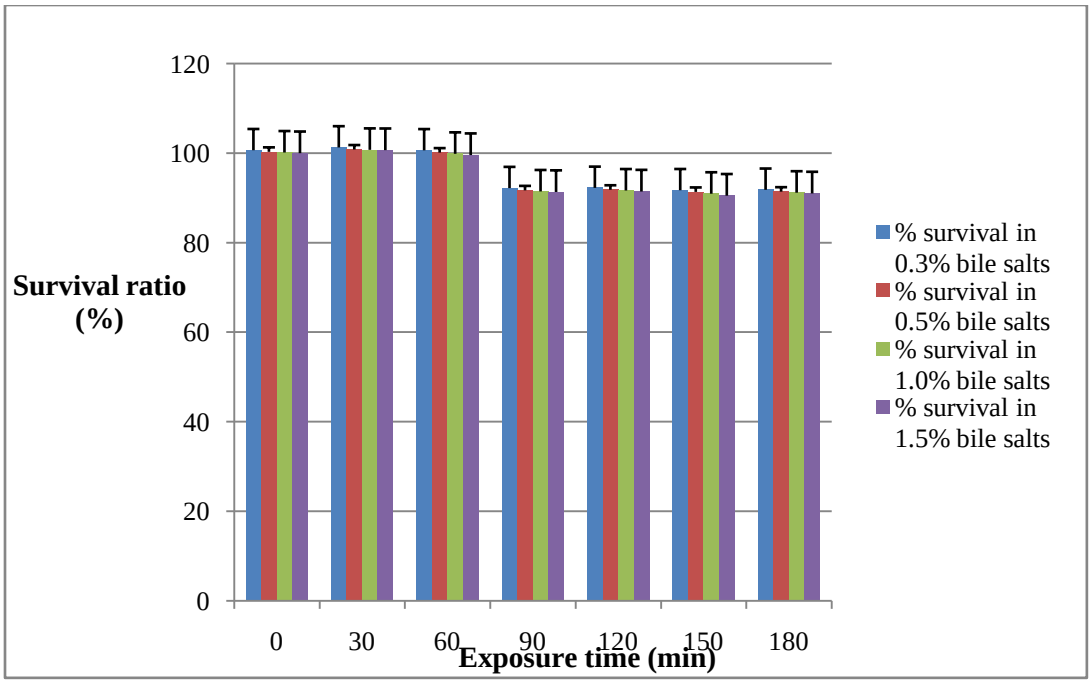

Figure 3.9: Percentage survival ratios of *Lactobacillus pentosus* in 0.3, 0.5, 1.0 and 1.5 % bile salts. The error bars are standard deviation of duplicate experiments.

Cells of *Weissella confusa* could not survive in pH 2.0 + pepsin and pH 2.0 without pepsin. Therefore, pepsin did not have any effect on survival of cells at this pH. In pH 3.0 + pepsin, pH 3.0 without pepsin, pH 4.0 + pepsin and pH 4.0 without pepsin the cells survived up to the third hour (Figure 3.10). Cells of *Weissella confusa* survived up to three hours in 0.3% and 0.5%. But in 1.0 and 1.5% bile, the cells could not survive beyond the second hour (Figure 3.11).

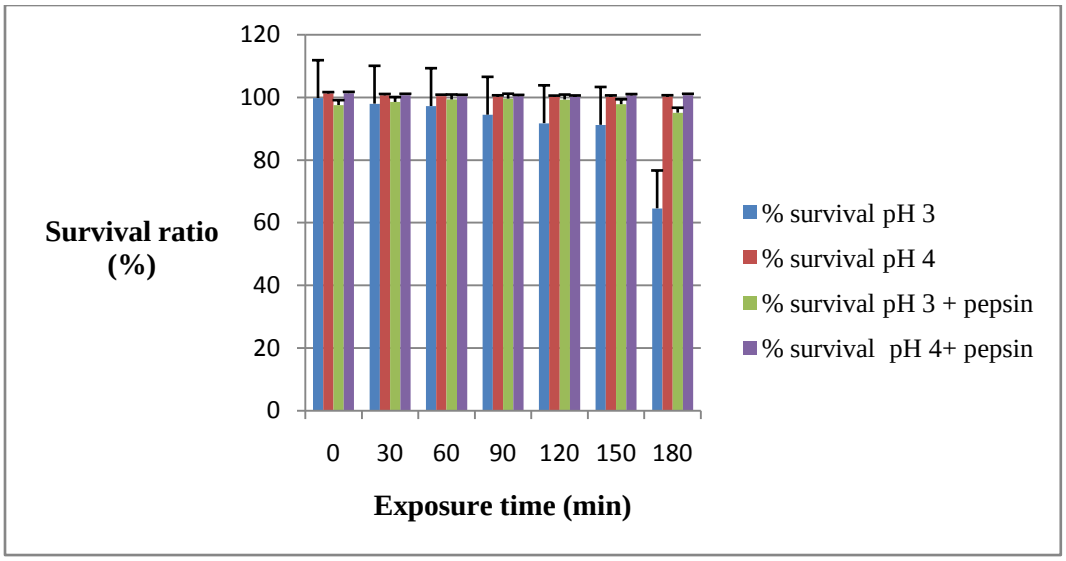

Figure 3.10: Percentage survival ratios of *Weisella confusa* at pH values of 3.0 and 4.0 with and without pepsin. The error bars are standard deviation of duplicate experiments.

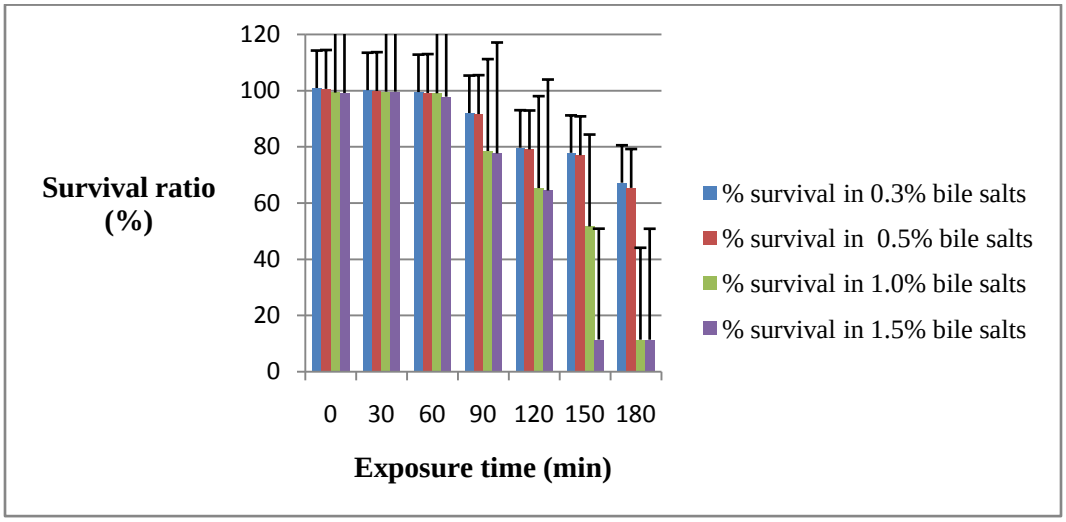

Figure 3.11: Percentage survival ratios of *Weisella confusa* in 0.3, 0.5, 1.0 and 1.5 % bile salts. The error bars are standard deviation of duplicate experiments.

Streptococcus salivarius subsp. *thermophilus* cells survived in pH 2.0 + pepsin up to the first hour but the cells could not survive in pH 2.0. Pepsin therefore had slight effect on survival at pH 2. In pH 3.0 + pepsin, pH 3.0 without pepsin, pH 4.0 + pepsin and pH 4.0 without pepsin the cells survived up to the third hour (Figure 3.12). In 0.3%, 0.5% and 1% bile concentrations, *Streptococcus salivarius*

subsp. *thermophilus* cells also survived up to the third hour. But in 1.5% bile, the cells could not survive beyond the second hour (Figure 3.13).

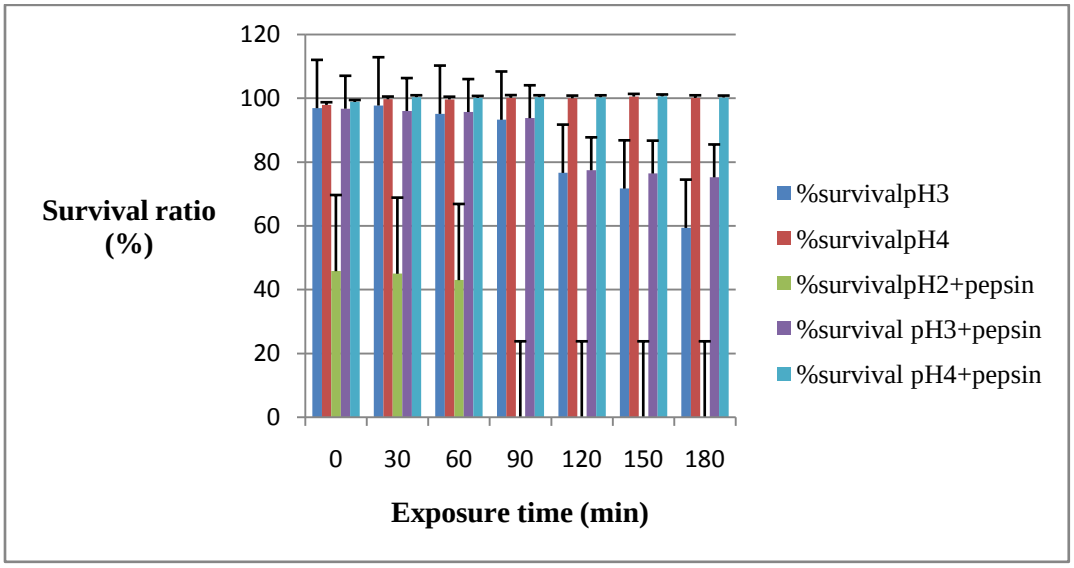

Figure 3.12: Percentage survival ratios of *Streptococcus salivarius* subsp. *thermophilus* at pH values of 2.0, 3.0 and 4.0 with and without pepsin. The error bars are standard deviation of duplicate experiments.

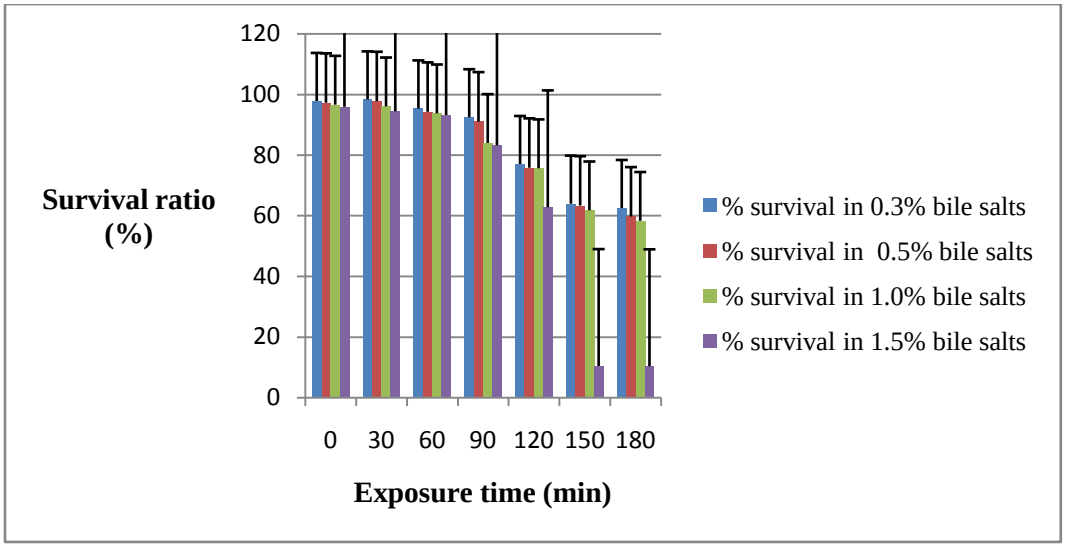

Figure 3.13: Percentage survival ratios of *Streptococcus salivarius* subsp. *thermophilus* in 0.3, 0.5, 1.0 and 1.5 % bile salts. The error bars are standard deviation of duplicate experiments.

Lactobacillus plantarum cells survive in pH 2.0 + pepsin up to the third hour but the cells only survive in pH 2.0 without pepsin at the initial zero minute. Pepsin had major effect on survival at pH 2. In pH 3.0 + pepsin, pH 3.0 without pepsin, pH 4.0 + pepsin and pH 4.0 without pepsin the cells survived the three hours of incubation (Figure 3.14). *Lactobacillus plantarum* cells also survived in all the bile salt concentrations of 0.3%, 0.5%, 1% and 1.5% (Figure 3.15).

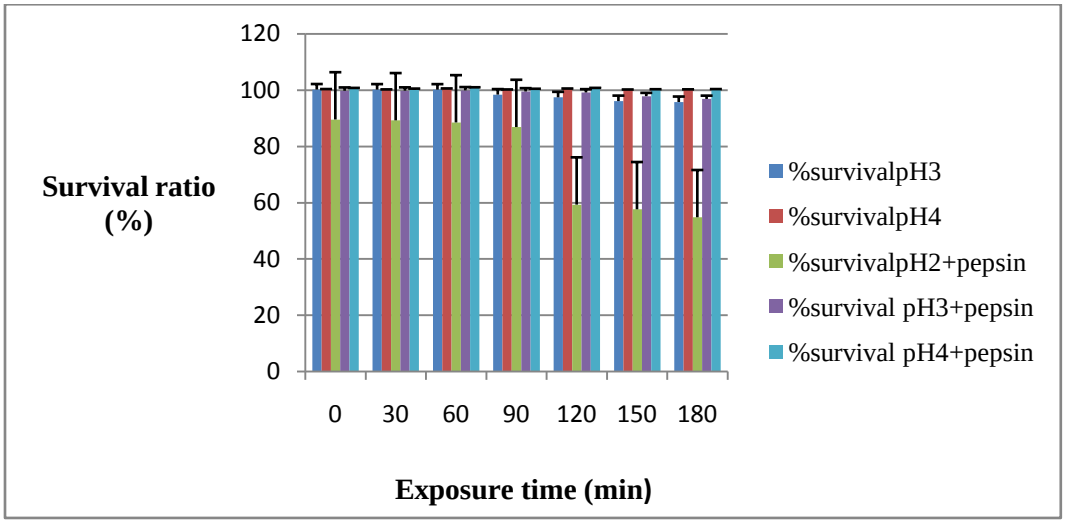

Figure 3.14: Percentage survival ratios of *Lactobacillus plantarum* at pH values of 2.0, 3.0 and 4.0 with and without pepsin. The error bars are standard deviation of duplicate experiments.

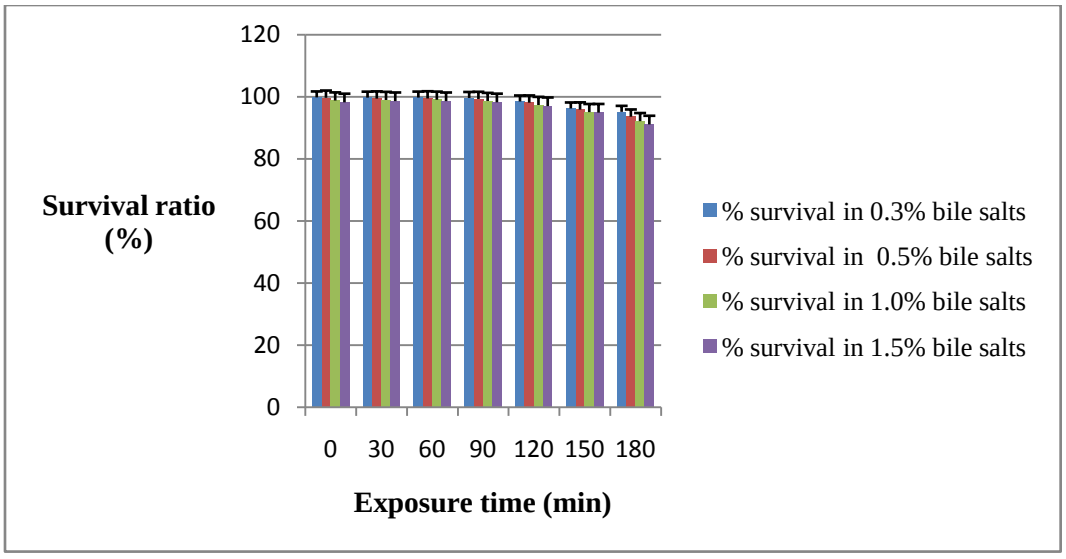

Figure 3.15: Percentage survival ratios of *Lactobacillus plantarum* in 0.3, 0.5, 1.0 and 1.5 % bile salts. The error bars are standard deviation of duplicate experiments.

Lactobacillus coryniformis cells survive in pH 2.0 + pepsin and pH 2.0 without pepsin up to 90 minutes. The cells survived in pH 3.0 + pepsin and pH 3.0 without pepsin, pH 4.0 + pepsin and pH 4.0 without pepsin up to the third hour (Figure 3.16). *Lactobacillus coryniformis* cells were also able to survive all the concentrations of bile salt of 0.3, 0.5, 1 and 1.5% (Figure 3.17).

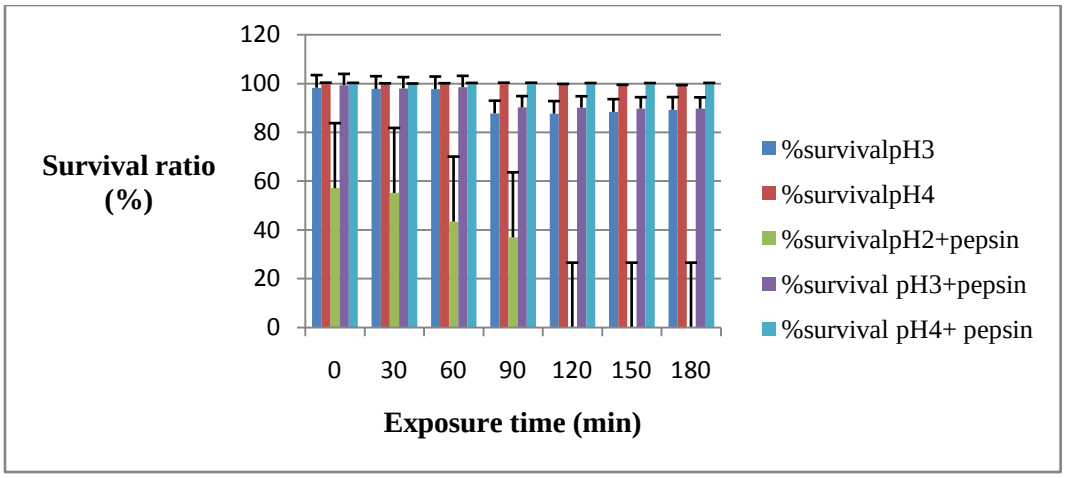

Figure 3.16: Percentage survival ratios of *Lactobacillus coryniformis* at pH values of 2.0, 3.0 and 4.0 with and without pepsin. The error bars are standard deviation of duplicate experiments.

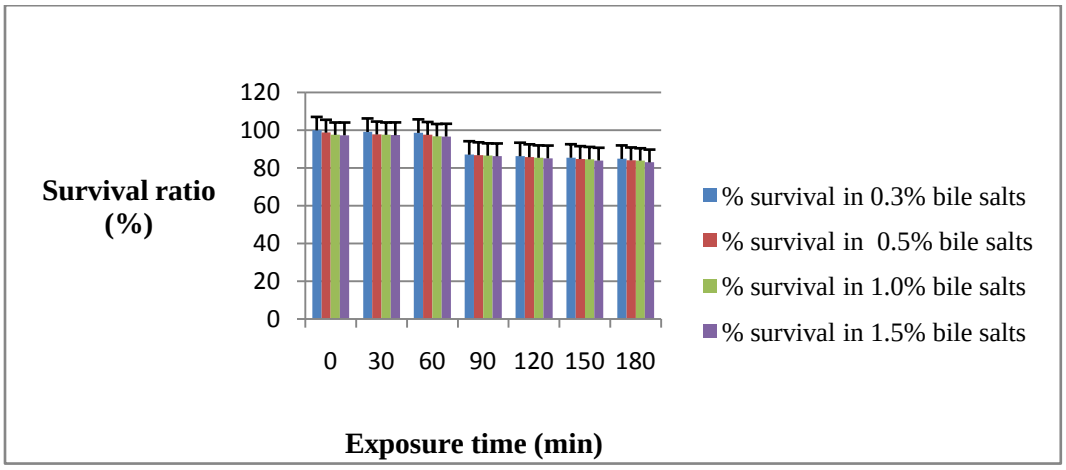

Figure 3.17: Percentage survival ratios of *Lactobacillus coryniformis* in 0.3, 0.5, 1.0 and 1.5 % bile salts. The error bars are standard deviation of duplicate experiments.

The cells of *Streptococcus cristatus* survived in pH 2.0 + pepsin up to the third hour but cells survived in pH 2.0 without pepsin for only 30 minutes. Pepsin therefore, had significant effect on survival of the cells at this pH. In pH 3.0 + pepsin, pH 3.0 without pepsin, pH 4.0 + pepsin and pH 4.0 without pepsin the cells survived the three hours (Figure 3.18). *Streptococcus cristatus* cells survived in all the bile concentrations of 0.3, 0.5, 1 and 1.5% (Figure 3.19).

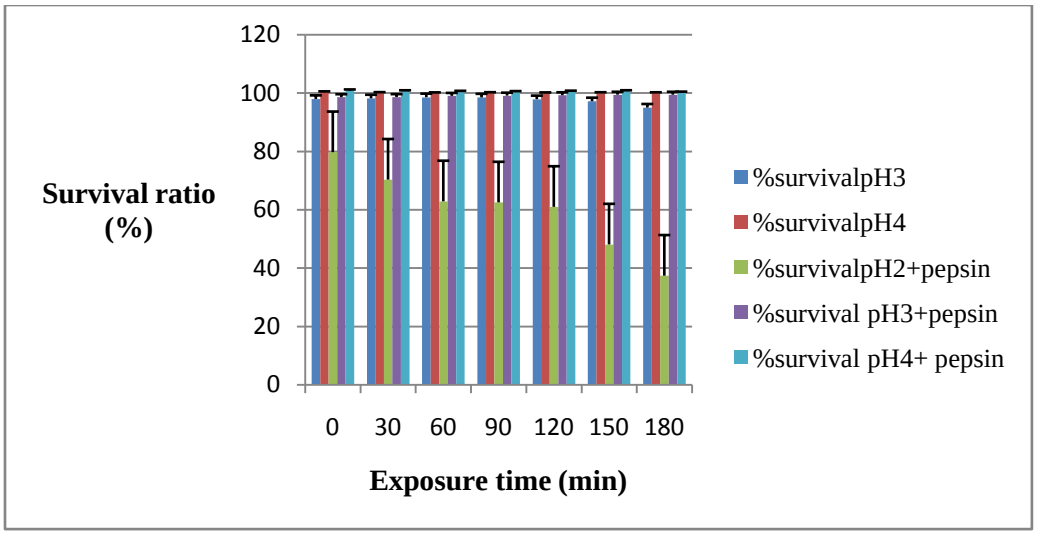

Figure 3.18: Percentage survival ratios of *Streptococcus cristatus* at pH values of 2.0, 3.0 and 4.0 with and without pepsin. The error bars are standard deviation of duplicate experiments.

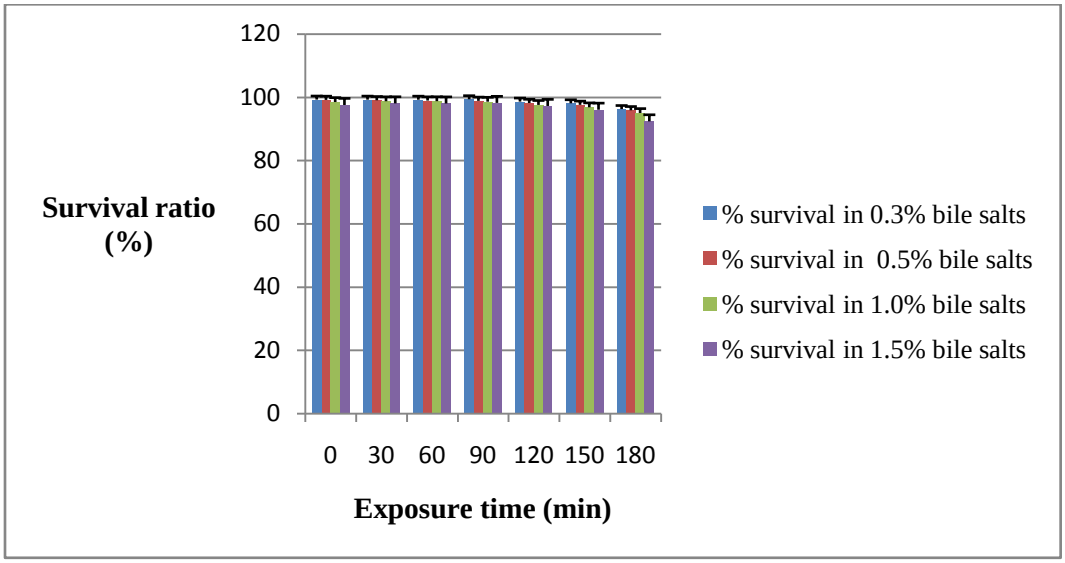

Figure 3.19: Percentage survival ratios of *Streptococcus cristatus* in 0.3, 0.5, 1.0 and 1.5 % bile salts. The error bars are standard deviation of duplicate experiments.

Leuconostoc mesenteroides subsp. *lactis* cells survived pH 2.0 + pepsin up to 2hrs 30 minutes but the cells survived in pH 2.0 for only 30 minutes. Pepsin had major effect on survival at this pH. The survivability of the cells in pH 3.0 + pepsin, pH 3.0 without pepsin, pH 4.0 + pepsin and pH 4.0 without pepsin was observed in

the three hours of incubation (Figure 3.20). The survivability of *Leuconostoc mesenteroides* subsp. *lactis* in all the bile salt concentrations of 0.3, 0.5, 1 and 1.5% was also observed for the three hours of incubation (Figure 3.21).

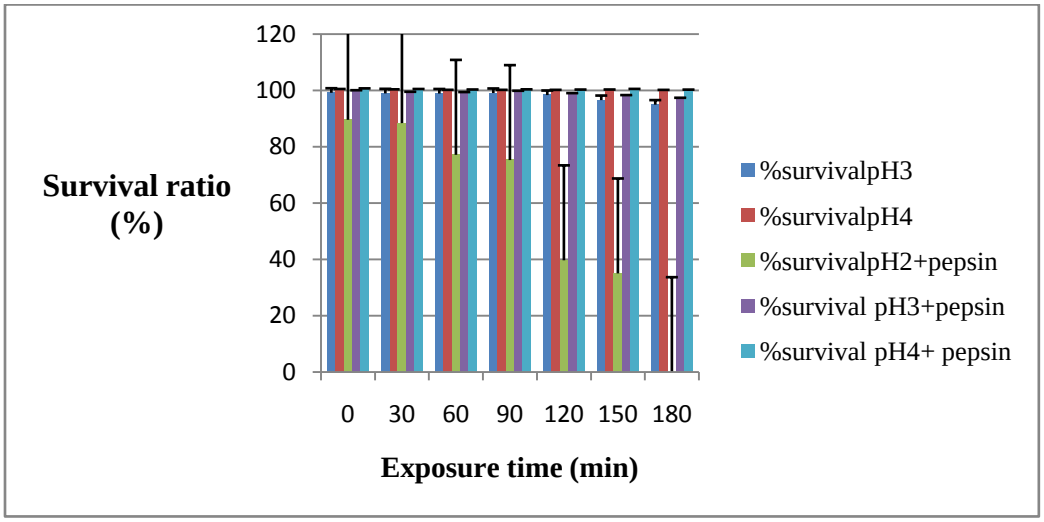

Figure 3.20: Percentage survival ratios of *Leuconostoc mesenteroides* subsp. *lactis* at pH values of 2.0, 3.0 and 4.0 with and without pepsin. The error bars are standard deviation of duplicate experiments.

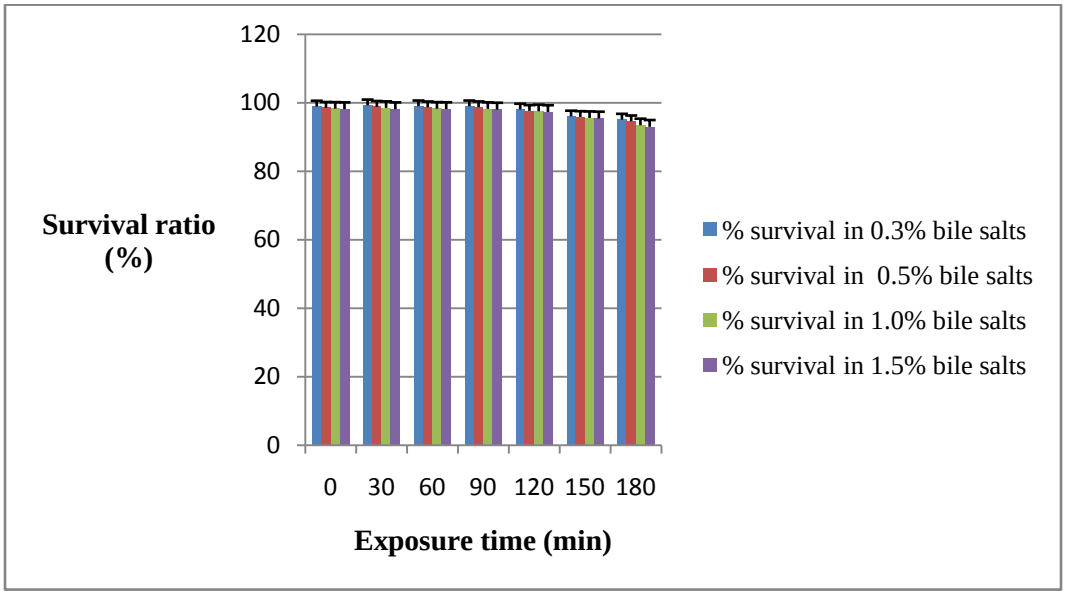

Figure 3.21: Percentage survival ratios of *Leuconostoc mesenteroides* subsp. *lactis* in 0.3, 0.5, 1.0 and 1.5 % bile salts. The error bars are standard deviation of duplicate experiments.

Enterococcus faecium cells survived pH 2.0 + pepsin up to 2hrs 30 minutes but the cells survived in pH 2.0 for the first hour. Pepsin therefore, had major effect on survival at this pH. The survivability of the cells in pH 3.0 + pepsin, pH 3.0 without pepsin, pH 4.0 + pepsin and pH 4.0 without pepsin was observed in the three hours of incubation (Figure 3.22). The survivability of *Enterococcus faecium* cells in all the bile salt concentrations of 0.3, 0.5, 1 and 1.5% for the three hours of incubation was also observed (Figure 3.23).

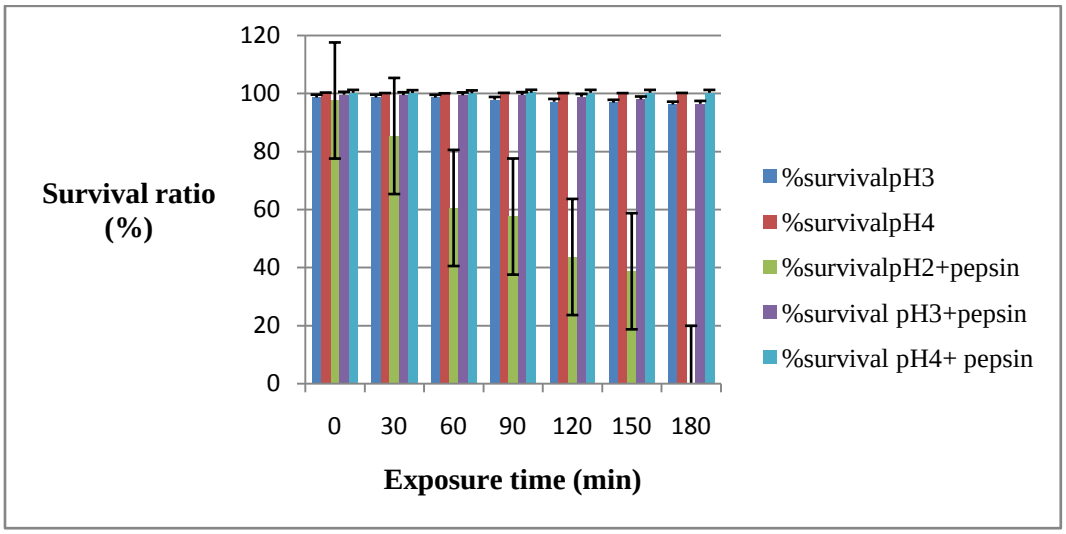

Figure 3.22: Percentage survival ratios of *Enterococcus faecium* at pH values of 2.0, 3.0 and 4.0 with and without pepsin. The error bars are standard deviation of duplicate experiments.

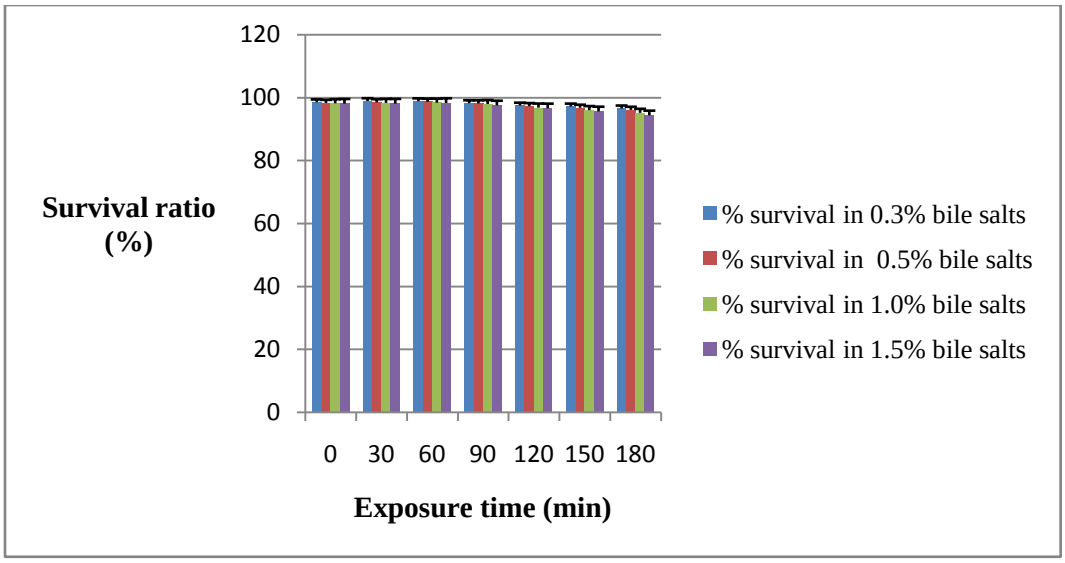

Figure 3.23: Percentage survival ratios of *Enterococcus faecium* in 0.3, 0.5, 1.0 and 1.5 % bile salts. The error bars are standard deviation of duplicate experiments.

3.4 Discussion

The tolerance to gastric acid and bile is considered a requirement for any probiotic bacteria. Acid and bile are likely to be the most antagonistic substances that affect the survivability and viability of LAB in the gut. The survivability of LAB in simulated pH and bile salts *in vitro* was therefore evaluated in this study. All the *Lactobacillus* species survived in pH 2.0 with pepsin for three hours of incubation, with the exception of *Lactobacillus coryniformis*, which survived for only 90 minutes. *Streptococcus cristatus* also survived in pH 2.0 with pepsin for the three hours. *Leuconostoc mesenteroides* subsp. *lactis* and *Enterococcus faecium* survived in pH 2.0 with pepsin for 150 minutes but *Streptococcus salivarius* subsp. *thermophilus* survived for only 60 minutes. *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Weissella paramesenteroides* and *Weissella confusa* could not survive in pH 2.0. All LAB survived in both pH 3.0 and pH 4.0 with and without pepsin, with the exception of *Leuconostoc mesenteroides* and

Weissella paramesenteroides, which failed to survive in pH 3.0 without pepsin. The ability of *Leuconostoc mesenteroides* and *Weissella paramesenteroides* to survive in pH 3.0 + pepsin may have been influenced by the protective effect of the pepsin enzyme. Survival of LAB in low pH is therefore confirmed by this research to be variable. According to Zhang *et al*. (2013), *Leuconostoc mesenteroides* subsp. *lactis* survived better in pH 3.0 with pepsin (4g/L) than in higher concentrations of pepsin (6,8 and 10g/L), confirming its excellent survival in this research at pH 3.0 in a concentration of 3g/L pepsin. The results of Hosseini *et al*. (2009) indicated that *Enterococcus faecium* survived better in pH 3.0 with pepsin than without, again validating the research herein reported. Bendali *et al*. (2011) reported survival of *Lactobacillus paracasei* in pH 2.0 with pepsin, this is in agreement with survival of *Lactobacillus* species in this research. Khalil *et al*. (2007) reported the tolerance of *Lactobacillus fermentum*, *Lactobacillus plantarum* and *Lactobacillus pentosus* to pH 3.0 and 0.3% bile, corroborating with the research herein reported.

Some LAB develop protective mechanisms that allow them to survive in acidic environments. The amino acid decarboxylase mechanism controls the pH of the bacterial environment by consuming hydrogen ions as part of the decarboxylation reaction, thus increasing the pH of the environment (Weeks and Sachs, 2001). Another mechanism is production of alkali, e.g. evolution of ammonia from urea through the action of urease (Weeks and Sachs, 2001). *Streptococcus salivarius* subsp. *thermophilus* possesses the urease gene enabling it to survive an acidic environment by neutralizing the acid (Cotter and Hill, 2003). Transporter-

dependent metabolic pathways favour the acid stress response in *Lactobacillus plantarum*, production of ATP at low pH promotes its survival in acidic conditions (Heunis *et al*. 2014).

Resistance to bile salts is another important criterion in the selection of probiotic bacteria. The small intestine and colon have high concentration of bile that has detrimental effects on bacterial cells. The cell membranes of bacteria are composed of lipid and fatty acids that are easily destroyed by bile salts (Meritt and Donaldson, 2009). Bile acid have antimicrobial effects on gut bacteria, deoxycholic acid (DCA) and cholic acid (CA) are potent antimicrobial compounds (Meritt and Donaldson, 2009). The entire LAB examined in this research survived the critical 0.3% bile salt concentration. They also survived concentration of 0.5%, however some LAB could not resist bile concentration of 1% and 1.5%. The 0.3% bile salt concentration is the critical concentration that any potential probiotic bacteria must tolerate (Shehata *et al*. 2016).

All 11 LAB were to some degree tolerant to pH values between 2.0 and 4.0 and to concentrations of bile between 0.3 and 1.5%. The gastric emptying time of breast-fed infants is 47 minutes faster than that of formula-fed infants, which is 65 minutes (Van *et al*. 1999). Therefore, beneficial LAB could easily pass to the colon and because of the rapid passage to the colon and relatively high pH (5.5 to 7) of the infant's stomach, beneficial LAB could improve the health and wellbeing of breast-fed babies.

3.5 Conclusion

The LAB evaluated in this chapter demonstrated properties that could possibly enable them to be used as probiotic bacteria for both babies and adults. Survival in acidic and bile solutions is among the hurdles that potential probiotic bacteria must overcome. The pepsin enzyme has enhanced tolerance of some of the screened LAB to very low pH. This study has contributed to the knowledge of the acid and bile resistance of some LAB that have been identified in samples of Nigerian human breast milk.

CHAPTER FOUR: ANTIMICROBIAL ACTIVITY OF LACTIC ACID BACTERIA

4.1 Introduction

The upsurge in antibiotic resistance among bacteria and consequent increasing failure of antibiotics to treat microbial diseases has prompted more research into alternative antimicrobial compounds. Promising and effective antimicrobial substances are now being investigated. Many reports exist on antimicrobial compounds produced by lactic acid bacteria (LAB) (Jacobsen *et al*. 1999, Hernandez *et al*. 2005, Ammor *et al*. 2006, Suskovic *et al*. 2010, Jara *et al*. 2011 and Leite *et al*. 2015). The antimicrobial activity of LAB has been associated with production of potent metabolites that include: organic acids (lactic and acetic acids), carbon dioxide, ethanol, hydrogen peroxide and bacteriocins (Hernandez *et al*. 2005, Ammor *et al*. 2006 and Suskovic *et al*. (2010). Moreover, the antimicrobial substances produced by LAB have been categorised into two groups: firstly, low molecular mass substances of less than 1000 Da and secondly, high molecular mass substances greater than 1000 Da (Ammor *et al*. 2006).

4.1.2 Low molecular mass antimicrobials

Low molecular mass antimicrobials constitute a diverse range of metabolites produced by LAB. Organic acids are the most important and potent antimicrobials produced by these bacteria (Salminen and Wright, 1998, Ammor *et al*. 2006 and Leite *et al*. 2015). The inhibitory and antagonistic effect of organic acids is attributed to reduction of intracellular pH and dissipation of membrane potential. Additionally, hydrogen peroxide has strong oxidizing effects on microbial cells and a destructive effect on cell proteins and molecular structure (Ammor *et al*. 2006). Reuterin and reutericyclin produced by some strains of *Lactobacillus*

reuterii have broad spectrum effects on both Gram positive and Gram negative bacteria, as well as fungi and protozoa (Suskovic *et al*. 2010). Reuterin and Reutericyclin play a vital role in the probiotic effects of some species of *Lactobacillus reuterii*, especially in the prevention of diarrhoea. Moreover, these potent antimicrobial substances are bactericidal against pathogenic microorganisms, they are also important in food preservation (Ammor *et al*. 2006).

Other low molecular mass antimicrobials include cyclic dipeptides produced by *Lactobacillus plantarum* and *Lactobacillus pentosus.* Three (3)-phenyllactic acid and 4-hydroxyphenyllactic acid are produced by *Lactobacillus plantarum, Lactobacillus rhamnosus, Lactobacillus acidophilus* and *Leuconostoc mesenteroides* subsp. *mesenteroides*. Benzoic acid, methylhydantoin and mevalonolactone are also produced by *Lactobacillus plantarum*, which have antimicrobial effects against fungi and Gram positive bacteria (Hernandez *et al*. 2005, Ammor *et al*. 2006 and Suskovic *et al*. 2010).

4.1.2 High molecular mass antimicrobials

Potent bacteriocins and other antibacterial proteinaceous substances with broad and narrow spectrum activity against pathogenic microbes are elaborated by LAB. Bacteriocins are ribosomally-synthesized peptides and proteins and are classified into three groups, firstly lantibiotics (lanthionine-containing bacteriocin) including nisin, lactocin, and mersacidin. The second group is the non-lanthionine-containing bacteriocins including pedocin (sakacin, leucocin and curvacin), lactococcin (plantaricin and lacticin) and acidocin (enterocin and

reuterin). The third group comprises bacteriolysins such as lysostaphin, enterolysin, helvetican and helveticin (Hernandez *et al*. 2005, Ammor *et al*. 2006 and Suskovic *et al*. 2010).

Various laboratory techniques are used in assessing antimicrobial activity of LAB. But the most frequently used methods are the agar spot test and agar well diffusion assay. The aim of the study described in this chapter was to investigate the antimicrobial activity of some of the identified LAB against four pathogenic bacteria. Ability of LAB to produce antimicrobial metabolites that will inhibit the growth of pathogenic bacteria is amongst the criteria for selection of probiotic LAB. The specific objectives of this study were:

- To evaluate the antimicrobial activity of the LAB using the unbuffered and buffered agar spot tests.
- To assess the antimicrobial activity of the LAB using the agar well diffusion assay.
- To investigate sensitivity of LAB inhibitory compounds to heat, proteolytic enzymes (proteinase K and protease) and catalase.

The LAB selected for investigation were:

- *Enterococcus faecium* (A1a[Mc])
- *Lactobacillus coryniformis* (T3b[Mc])
- *Lactobacillus fermentum* (O3[M])
- *Lactobacillus pentosus* (T2a[M])
- *Lactobacillus plantarum* (P3[M])

- *Leuconostoc mesenteroides* subsp. *lactis* (R1[M])
- *Leuconostoc mesenteroides* subsp. *mesenteroides* (C2c[Mc])
- *Streptococcus cristatus* (Q1[M])
- *Streptococcus salivarius* subsp. *thermophilus* (S2b[Mc])
- *Weissella confusa* (M2c[M])
- *Weissella paramesenteroides* (D1[Mc])

Each of the LAB selected represent other identified LAB of the same genus and species. The selection was based on similarity in their rep-PCR profiles. The indicator organisms used to demonstrate antimicrobial effects by the LAB were: *Escherichia coli* NCTC 12900, *Salmonella* Enteritidis DT124, *Staphylococcus aureus* CMCC 1930 and *Bacillus cereus* NCFB 13507, derived from the London Metropolitan University Microbiology Research Unit (MRU) culture collection.

4.2 Materials and methods

The evaluation of the antimicrobial potential of the 11 selected LAB was carried out according to the methods of Hernandez *et al*. (2005), Kizerwetter-Swida and Binek, (2005), Ammor *et al*. (2006), Kivanc *etal*. (2011), Al-Otaibi (2012) and Anyogu *et al*. (2014). The antimicrobial activity was evaluated using the agar spot test and the agar well diffusion assay. The indicator organisms were the four pathogenic bacteria provided by the Microbiology Research Unit (MRU). The test organisms were the 11selected LAB.

4.2.1 Inoculum preparation

Pathogenic bacterial inocula (indicator organisms)

Escherichia coli NCTC 12900, *Salmonella* Enteritidis DT124, *Staphylococcus aureus* CMCC 1930 and *Bacillus cereus* NCFB 13507 were derived from the Microbiology Research Unit (MRU) culture collection for use as indicator organisms.

Colonies of a 24 h culture of each indicator bacterium grown aerobically on nutrient agar at 37 ^{o}C were dispersed in 1 ml maximum recovery diluent (MRD). The suspension was adjusted to 0.5 McFarland standard to give an approximate concentration of 10^{8} cfu/ml. These microbial suspensions were used as the indicator inocula for the experiments.

LAB inocula

Colonies of a 24 h culture of the isolate grown on MRS agar anaerobically at 37 ^{o}C was sub-cultured into 1 ml maximum recovery diluent (MRD). The suspension was adjusted to 0.5 MacFarland standard, giving an approximate concentration of 10^{8} cfu/ml. This was used for the spot test.

4.2.2 Unbuffered agar spot test

The agar spot test was used for preliminary assessment of antagonistic effects of the 11 selected LAB against *Escherichia coli, Salmonella* Enteritidis, *Staphylococcus aureus* and *Bacillus cereus* (indicator organisms). An aliquot of 5 µl of LAB inocula was spotted on MRS agar and the plates were allowed to dry for one hour at room temperature. The plates were incubated at 37 ^{o}C anerobically

for 24 h to initiate growth of the test organism. The colonies of developing test organism were then overlaid with 10 ml soft Tryptone Soya Agar (soft TSA), the agar was prepared by adding 0.8% (w/v) plain agar (LP0012, Oxoid, UK) to Tryptone Soya Broth (TSB; CM0129, Oxoid, UK). After autoclaving this was retained in a molten state at 45 ^{o}C and 100 µl (10^{8} cfu) of indicator organism were added. The overlaid plates were incubated inverted at 37 ^{o}C aerobically for another 24 h. The zones of inhibition of the indicator organism by the test organism were measured in mm using a ruler.

4.2.3 Buffered agar spot test

To rule out the effect of the acid content of MRS agar on inhibition of growth of the indicator organisms, the acid in the MRS agar medium was neutralised. MRS agar containing 2g/L of sodium bicarbonate was prepared for the test organisms and the procedure described in Section 4.1.2 was followed.

4.2.4 Agar well diffusion assay

The LAB that indicated clear zones of inhibition in the agar spot test were further assessed for antimicrobial activity using the agar well diffusion assay (AWDA). A 24 h culture of each LAB in MRS broth (CM0359, Oxoid UK) was centrifuged at 1000 x g for 10 min at 4 ^{o}C. The cell-free supernatant was filtered through a 0.2 µm sterile filter and this was used for the experiment. Twenty milliliters soft Tryptone Soya Agar (TSA) which was prepared by adding 0.8% (w/v) plain agar (LP0012, Oxoid, UK) to Tryptone Soya Broth (TSB; CM0129, Oxoid, UK) was used. After autoclaving the agar, it was retained in a molten state at 45 ^{o}C and 100 µl (10^{8} cfu) of each indicator organism were added and was poured into Petri

dishes. When the agar solidified, wells were created using sterile 200-1000 µl pipette tip. The wells were filled with 100 µl filtered supernatant of the LAB. The plates were kept at 4 ^{o}C for 3 hours to allow diffusion of supernatant of the LAB. Plates were incubated inverted at 37 ^{o}C aerobically for 24 h. The zones of inhibition of the indicator organism by the test organism were measured in mm using a ruler.

4.2.5 Agar well diffusion assay with concentrated buffered and concentrated unbuffered supernatant

A 24 h culture of LAB in MRS broth (CM0359, Oxoid UK) was prepared as above. A volume of 10 mls of cell-free supernatant with original pH of 4 to 4.4 was concentrated to 1g by freeze drying (Heto PowerDry PL3000 Freeze Dryer, Thermo Electron Corporation, UK) at -56 ^{o}C and 0.16 hpa for 48 h. The concentrated supernatant was diluted fivefold (1/5, 1g supernatant into 4ml MRS broth). To assess whether inhibitory effect of antimicrobial substances was not organic acid, part of the concentrated supernatant was neutralized to pH 6.7 using sterile 1 M sodium hydroxide (NaOH). The buffered supernatant was also used for the experiment. The procedure described as above for agar well diffusion was followed.

4.2.6 Thermal heat treatment

The concentrated cell free supernatants of the LAB isolates that showed positive results in the agar well diffusion assay were subjected to heat treatment using Techne Dri-Block (DB-2D, Thistle Scientific, UK). A volume of 1000 µl of each LAB supernatant was placed in the heating block (63 ^{o}C for 30 min) and samples

cooled to 4 ^{o}C. The residual antibacterial activity of the heat treated cell-free supernatants was assessed using the agar diffusion assay. The procedure described in above for agar well diffusion was followed.

4.2.7 Effect of proteolytic enzymes

To assess if inhibition of growth of indicator organisms is as a result of production of peptides, sensitivity of antimicrobials to proteinase K 1mg/ml (BP 1700-100, Fischer Scientific, UK) and protease 1 mg/ml (P5147, Sigma, UK) was evaluated. An aliquot of 100 µl of each enzyme (filter sterilised) was added to 900 µl supernatant and the samples were incubated at 30 ^{o}C for 1 h. One well containing supernatant without the enzymes was used as control. The residual activity was determined using the agar well diffusion assay. The procedure described as above for agar well diffusion was followed.

4.2.8 Effect of catalase

To assess if inhibition of growth of indicator organisms is as a result of production of hydrogen peroxide H_2O_2, 100 µl (filter sterilised) catalase enzyme (C9322 Sigma, UK) at a final concentration of 1mg/ml was added to 900 µl supernatant. One well containing supernatant without catalase was used as control. The residual activity was determined using the agar well diffusion assay. The procedure described as above for agar well diffusion was followed.

4.3 Results

4.3.1 Unbuffered and buffered agar spot test

All 11 LAB produced antibacterial metabolites that inhibited growth of all four indicator bacteria in both the unbuffered and buffered agar spot tests. The strongest zones of inhibitions were seen in the unbuffered agar spot test (Table 4.1 and Figure 4.1). In the buffered agar spot test, *Lactobacillus pentosus, Streptococcus salivarius* subsp. *thermophilus* and *Lactobacillus plantarum* exhibited the strongest zones of inhibition against *Bacillus cereus* as determined by measuring the zones (Figure 4.2). *Leuconostoc mesenteroides* subsp. mesenteroides, *Lactobacillus pentosus, Weissella confusa, Leuconostoc mesenteroides* subsp. *lactis* and *Lactobacillus coryniformis* showed the strongest inhibition against *Staphylococcus aureus*. *Weissella paramesenteroides, Lactobacillus pentosus, Weissella confusa, Lactobacillus plantarum, Leuconostoc mesenteroides* subsp. *lactis* and *Lactobacillus coryniformis* exhibited the strongest inhibition against *Salmonella enteritidis*. Only *Lactobacillus fermentum* strongly inhibited *Escherichia coli* (Table 4.2). The agar spot test demonstrates ability of LAB cell cultures to inhibit growth of pathogenic bacteria.

Table 4.1 Antibacterial activity of LAB against indicator bacteria using unbuffered agar spot test.

Lactic acid bacteria	*B. cereus*	*E. coli*	*Staph. aureus*	*S.* Enteritidis
Leuconostoc mesenteroides subsp. *mesenteroides*	+++	+++	+++	+++
Weissella paramesenteroides	+++	+++	++	+++
Lactobacillus fermentum	+++	+++	+++	+++
Lactobacillus pentosus	+++	+++	+++	+++
Weissella confusa	+++	+++	+++	+++
Streptococcus salivarius subsp. *thermophilus*	+++	+++	++	+++
Enterococcus faecium	++	+++	++	++
Lactobacillus plantarum	+++	+++	+++	+++
Leuconostoc mesenteroides subsp. *lactis*	+++	+++	++	+++
Lactobacillus coryniformis	+++	+++	+++	+++
Streptococcus cristatus	+++	+++	++	++

(-) = no inhibition

(+) = Zone of inhibition < 10 mm

(++) = Zone of inhibition between 10-20 mm

(+++) = Zone of inhibition > 20 mm

Table 4.2 Antibacterial activity of LAB against indicator bacteria using buffered agar spot test.

Lactic acid bacteria	*B. cereus*	*E. coli*	*Staph. aureus*	*S.* Enteritidis
Leuconostoc mesenteroides subsp. *mesenteroides*	++	++	+++	++
Weissella paramesenteroides	++	++	++	+++
Lactobacillus fermentum	++	+++	++	++
Lactobacillus pentosus	+++	++	+++	+++
Weissella confusa	++	++	+++	+++
Streptococcus salivarius subsp. *thermophilus*	+++	++	++	++
Enterococcus faecium	++	++	++	++
Lactobacillus plantarum	+++	++	++	+++
Leuconostoc mesenteroides subsp. *lactis*	++	++	+++	+++
Lactobacillus coryniformis	++	++	+++	+++
Streptococcus cristatus	++	++	++	++

(-) = no inhibition

(+) = Zone of inhibition < 10 mm

(++) = Zone of inhibition between 10-20 mm

(+++) = Zone of inhibition > 20 mm

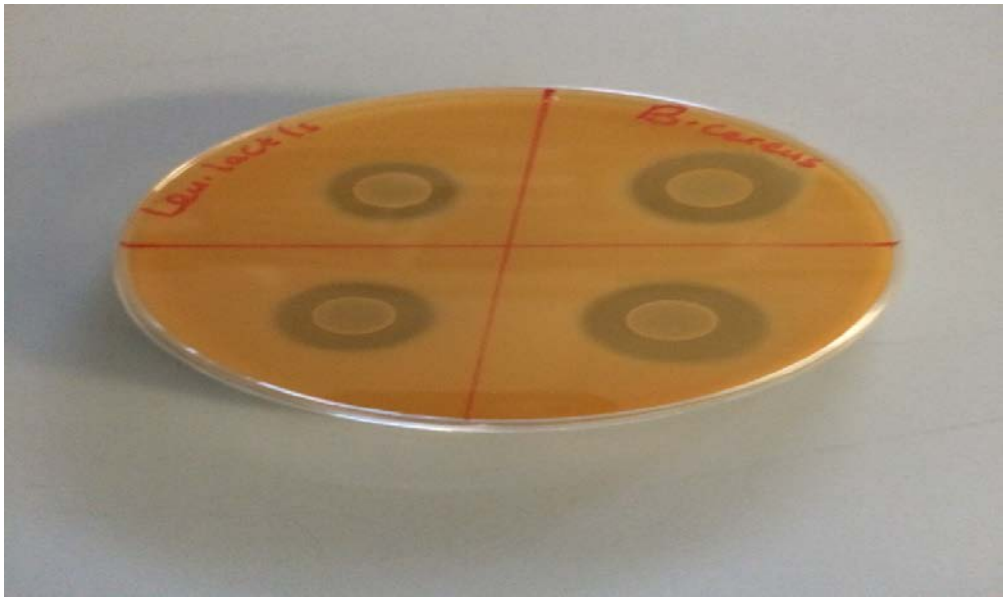

Figure 4.1: Antimicrobial activity of *Leuconostoc mesenteroides* subsp. *lactis* against *Bacillus cereus* in unbuffered agar spot test

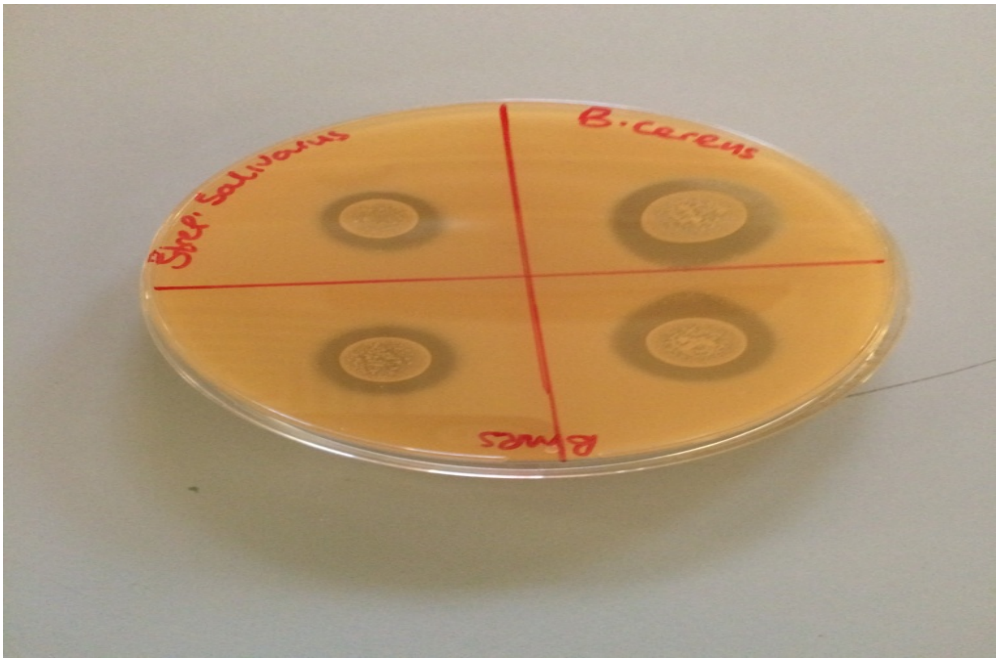

Figure 4.2: Antimicrobial activity of *Streptococcus salivarius* subsp. *thermophilus* against *Bacillus cereus* in buffered agar spot test

4.3.2 Agar well diffusion assay

The initial results of the agar well diffusion assay revealed ability of *Lactobacillus coryniformis* and *Lactobacillus pentosus* to moderately inhibit *E. coli*, *B. cereus* and *S.* Enteritidis but no inhibition was seen against *Staph.aureus.* Zones of inhibition were also seen in *Lactobacillus fermentum, Lactobacillus plantarum and Streptococcus cristatus* against *B. cereus*. No inhibition was seen against *Staph. aureus*, *E. coli*, and *S.* Enteritidis. *Streptococcus salivarius* subsp. *thermophilus, Weissella confusa*, *Leuconostoc mesenteroides* subsp. *lactis, Weissella paramesenteroides, Enterococcus faecium* and *Leuconostoc mesenteroides* subsp. *mesenteroides* did not exhibit antimicrobial activity against the indicator organisms (Table 4.3). The agar well diffusion assay (AWDA) demonstrates antagonistic effects of cell-free supernatants of LAB against pathogenic bacteria.

Table 4.3 Antibacterial activity of cell free supernatant against indicator bacteria using agar well diffusion assay.

Lactic acid bacteria	*B. cereus*	*E. coli*	*Staph. aureus*	*S.* Enteritidis
Leuconostoc mesenteroides subsp. *mesenteroides*	-	-	-	-
Weissella paramesenteroides	-	-	-	-
Lactobacillus fermentum	+	-	-	-
Lactobacillus pentosus	++	++	-	++
Weissella confusa	-	-	-	-
Streptococcus salivarius subsp. thermophilus	-	-	-	-
Enterococcus faecium	-	-	-	-
Lactobacillus plantarum	++	-	-	-
Leuconostoc mesenteroides subsp. *lactis*	-	-	-	-
Lactobacillus coryniformis	++	+	-	++
Streptococcus cristatus	++	-	-	-

(-) = no inhibition

(+) = Zone of inhibition < 10 mm

(++) = Zone of inhibition between 10-20 mm

(+++) = Zone of inhibition > 20 mm

4.3.3 Agar well diffusion assay with concentrated unbuffered and concentrated buffered supernatant

Since not all of the LAB cell free supernatants inhibited growth of pathogenic bacteria, the cell free supernatants were concentrated in order to obtain higher concentration of antimicrobial compounds. It was observed that *Lactobacillus pentosus, Lactobacillus plantarum, Lactobacillus coryniformis* and *Streptococcus cristatus* exhibited moderate inhibition against all the indicator bacteria. *Weissella confusa* showed inhibition against *E. coli, B. cereus* and *Staph. aureus. Lactobacillus fermentum* exhibited inhibition against *B. cereus* and *E. coli* while *Weissella paramesenteroides* and*Leuconostoc mesenteroides* subsp.*mesenteroides* showed inhibition against *Staph. aureus* (Table 4.4 and Figure 4.3). *Streptococcus salivarius* subsp. *thermophilus, Leuconostoc mesenteroides* subsp. *lactis, and Enterococcus faecium* did not exhibit any inhibition against the indicator bacteria. When neutralised concentrated cell free supernatants of the LAB were dispensed into agar wells, no zones of inhibition were seen against the indicator bacteria after incubation.

Table 4.4 Antibacterial activity of cell free concentrated supernatant (5 fold) against indicator bacteria using agar well diffusion assay.

Lactic acid bacteria	*B. cereus*	*E. coli*	*Staph. aureus*	*S.* Enteritidis
Leuconostoc mesenteroides subsp. *mesenteroides*	-	-	+	-
Weissella paramesenteroides	-	-	+	-
Lactobacillus fermentum	+	++	-	-
Lactobacillus pentosus	++	++	++	++
Weissella confusa	++	+	+	-
Streptococcus salivarius subsp. *thermophilus*	-	-	-	-
Enterococcus faecium	-	-	-	-
Lactobacillus plantarum	++	++	++	++
Leuconostoc mesenteroides subsp. *lactis*	-	-	-	-
Lactobacillus coryniformis	++	++	++	++
Streptococcus cristatus	++	++	++	++

(-) = no inhibition

(+) = Zone of inhibition < 10 mm

(++) = Zone of inhibition between 10-20 mm

(+++) = Zone of inhibition > 20 mm

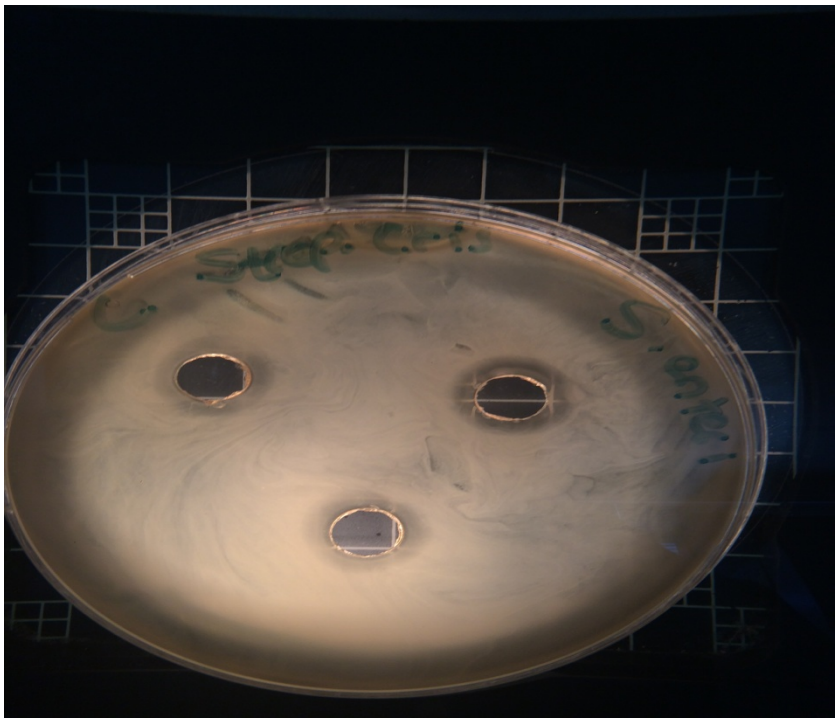

Figure 4.3: Antimicrobial activity of concentrated cell free supernatant of *Streptococcus cristatus* against *Salmonella* Enteritidis in agar well diffusion assay.

4.3.4 Thermal heat treatment

When concentrated cell free supernatants of LAB were heated at 63 ^{o}C for 30 min, *Lactobacillus pentosus, Lactobacillus plantarum, Lactobacillus coryniformis* and *Streptococcus cristatus* also exhibited inhibition against all indicator bacteria. But cell free supernatants of *Weissella paramesenteroides, Lactobacillus fermentum, Leuconostoc mesenteroides* subsp. *mesenteroides* and *Weissella confusa* did not have any antimicrobial activity against indicator bacteria (Table 4.5 and Figure 4.4).

Table 4.5 Effect of heat (63 ^{o}C for 30 min) on antimicrobial activity of concentrated cell free supernatant (5 fold) against indicator bacteria using agar well diffusion assay.

Lactic acid bacteria	*B. cereus*	*E. coli*	*Staph. aureus*	*S.* Enteritidis
Lactobacillus pentosus	++	++	+	++
Lactobacillus plantarum	++	++	++	++
Lactobacillus coryniformis	+	++	+	+
Streptococcus cristatus	++	+	+	++
Weissella paramesenteroides	-	-	-	-
Lactobacillus fermentum	-	-	-	-
Leuconostoc mesenteroides subsp. *mesenteroides*	-	-	-	-
Weissella confusa	-	-	-	-

(-) = no inhibition

(+) = Zone of inhibition < 10 mm

(++) = Zone of inhibition between 10-20 mm

(+++) = Zone of inhibition > 20 mm

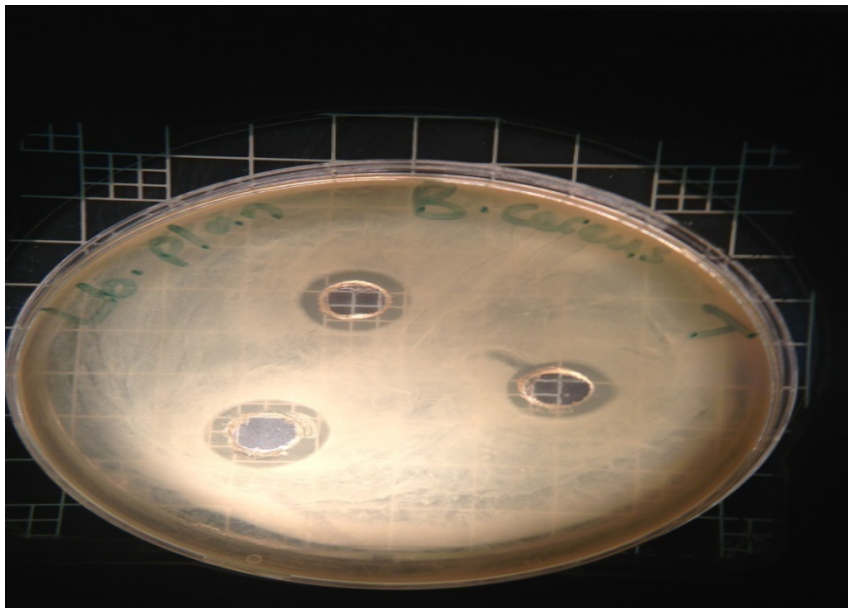

Figure 4.4: Antimicrobial activity of concentrated cell free supernatant (63 ^{o}C for 30 min) of *Lactobacillus plantarum* against *Bacillus cereus* in agar well diffusion assay.

4.3.5 Effect of proteolytic and catalase enzymes

Concentrated cell free supernatants of *Streptococcus cristatus* that were treated with proteinase K, protease and catalase enzymes all exhibited antimicrobial activity against all indicator bacteria. Similarly, *Lactobacillus pentosus, Lactobacillus plantarum* and *Lactobacillus coryniformis* showed inhibition against *B. cereus.* In *Lactobacillus pentosus*, inhibition was seen against *Staph.aureus* with proteinase K and protease but no inhibition was seen with catalase, the enzymes had effect on inhibition of *E. coli* and *S.* Enteritidis. In *Lactobacillus plantarum*, inhibition was seen in *E. coli* with proteinase K and protease but no inhibition was seen with catalase. No inhibition was also seen in *Staph. aureus* and *S.* Enteritidis. *Lactobacillus coryniformis* only exhibited antimicrobial activity against *Staph. aureus* with protease (Table 4.6 and Figures 4.5, 4.6 and 4.7).

Table 4.6 Effect of enzymes and neutralisation on antimicrobial activity of concentrated cell free supernatant (5 fold) against indicator bacteria using agar well diffusion assay.

LAB	Indicator bacteria	Proteinase K	Protease	Catalase	Neutralised CCFS
Lactobacillus pentosus	*B. cereus*	++	++	++	-
	E. coli	-	-	-	-
	Staph. aureus	++	++	-	-
	S. Enteritidis	-	-	-	-
Lactobacillus plantarum	*B. cereus*	++	++	++	-
	E. coli	++	++	-	-
	Staph. aureus	-	-	-	-
	S. Enteritidis	-	-	-	-
Lactobacillus coryniformis	*B. cereus*	++	++	++	-
	E. coli	-	-	-	-
	Staph. aureus	-	++	-	-
	S. Enteritidis	-	-	-	-
Streptococcus cristatus	*B. cereus*	++	++	++	-
	E. coli	++	++	++	-
	Staph. aureus	++	++	++	-
	S. Enteritidis	++	++	++	-

(-) = no inhibition

(+) = Zone of inhibition < 10 mm

CCFS = Concentrated cell free supernatant

(++) = Zone of inhibition between 10-20 mm

(+++) = Zone of inhibition > 20 mm

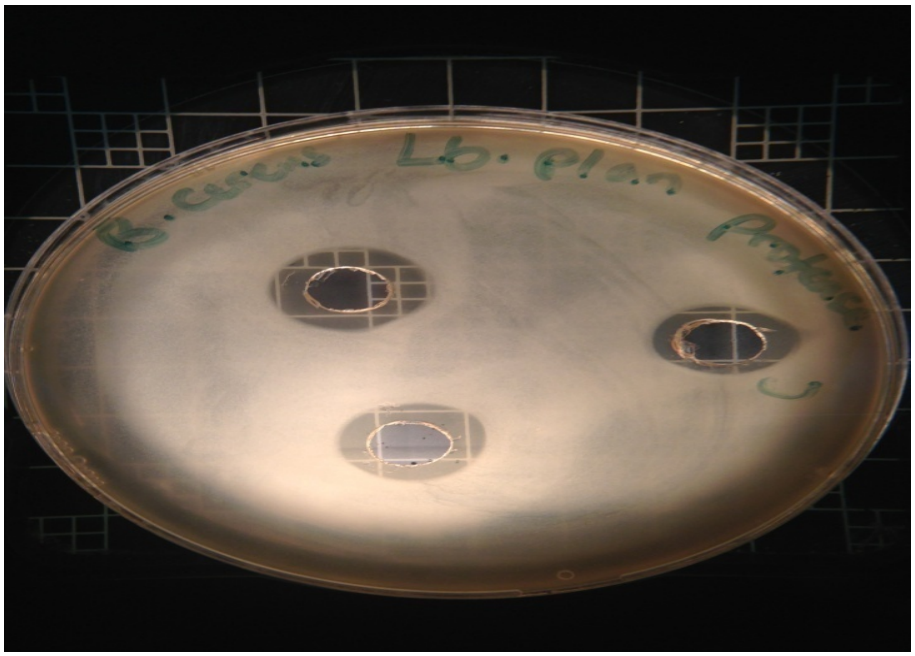

Figure 4.5: Antimicrobial activity of concentrated cell free supernatant (with protease) of *Lactobacillus plantarum* against *Bacillus cereus* in agar well diffusion assay.

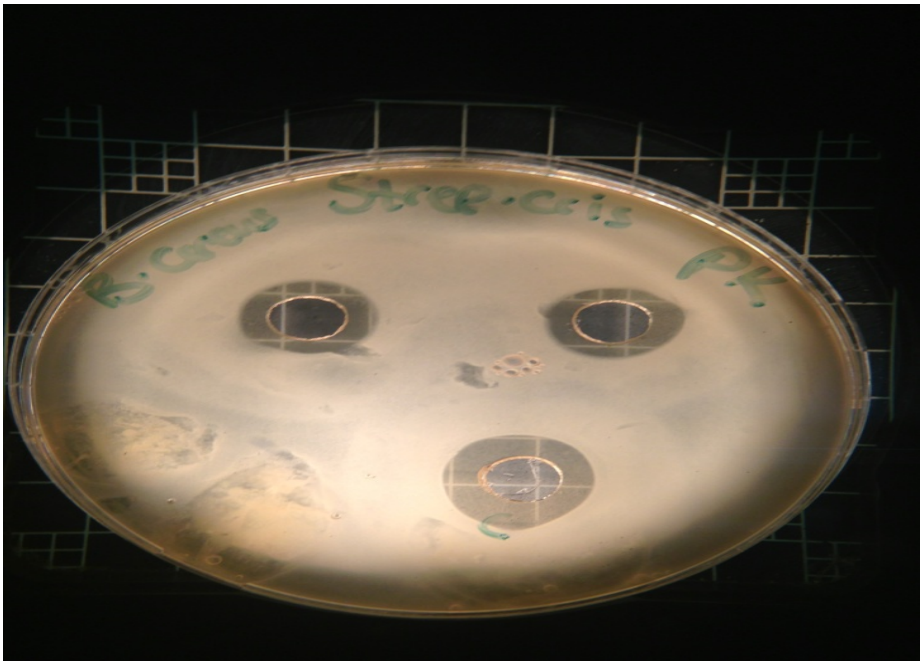

Figure 4.6: Antimicrobial activity of concentrated cell free supernatant (with proteinase K) of *Streptococcus cristatus* against *Bacillus cereus* in agar well diffusion assay.

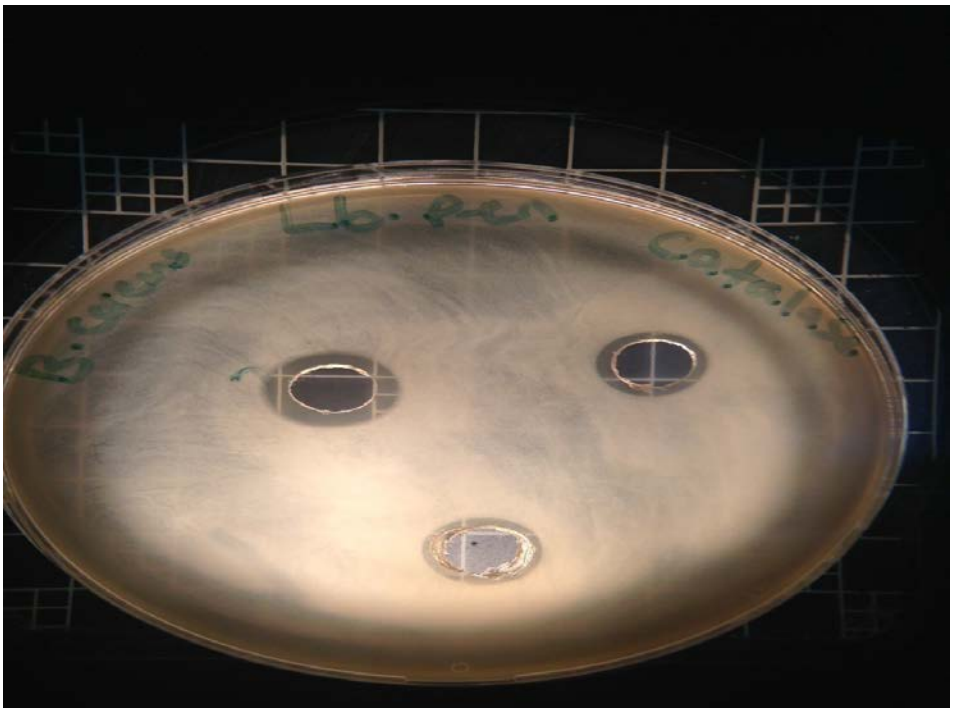

Figure 4.7: Antimicrobial activity of concentrated cell free supernatant (with catalase) of *Lactobacillus pentosus* against *Bacillus cereus* in agar well diffusion assay.

4.4 Discussion

The antimicrobial activity of LAB is important in the selection of potential probiotic LAB. In order to assess the probiotic potential of some of the identified LAB in this research, the study on antimicrobial activity of 11 selected LAB was carried out. The antimicrobial activity of LAB against four pathogenic bacteria was investigated and all 11 selected LAB inhibited growth of the four pathogens, in both the unbuffered and buffered agar spot tests, with the strongest inhibition recorded in the unbuffered agar spot test. According to Jara *et al*. (2011) and Leite *et al*. (2015), some members of the LAB produce bacteriocins and other antagonistic metabolites such as hydrogen peroxide, carbon dioxide and lactic and other acids. Moreover, reduction of pH due to the production of lactic acid is

detrimental to some pathogenic microbes (Jacobsen *et al*. 1999, Ammor *et al*. 2006, Pan *et al*. 2009 and Suskovic *et al*. 2010).

Similarly, Leite *et al*. (2015) studied probiotic potential of selected LAB (*Leuconostoc mesenteroides* subsp. *mesenteroides, Lactococcus lactis and Lactobacillus paracasei*) strains isolated from Beazalian kefir grains. They evaluated antimicrobial activity of the LAB against *Escherichia coli, Salmonella* Enteritidis, *Staphylococcus aureus and Listeria monocytogenes* using agar spot test. *Escherichia coli* and *Salmonella* Enteritidis were the most inhibited by the LAB, no inhibition was observed in *Staphylococcus aureus. Listeria monocytogenes* was inhibited by *Lactococcus lactis and Lactobacillus paracasei.* The inhibition of *Escherichia coli* and *Salmonella* Enteritidis by *Leuconostoc mesenteroides* subsp. *mesenteroides* in Leite *et al*. (2015) research corroborate with our result and our methodology. But, *Leuconostoc mesenteroides* subsp. *mesenteroides* in our study showed strong inhibition against *Staphylococcus aureus*.

The antimicrobial activity of LAB from fermented cassava was also studied by Anyogu *et al*. (2014) using the agar spot test and the well diffusion assay. Their results indicated that *Weissella confusa, Lactobacillus plantarum, Leuconostoc mesenteroides* subsp. *mesenteroides, Enterococcus faecium* and *Weissella paramesenteroides* exhibited zones of inhibition against *Escherichia coli, Salmonella typhimurium, Staphylococcus aureus* and *Bacillus cereus.* Although no inhibition in *Staphylococcus aureus* was seen in *Leuconostoc mesenteroides* subsp. *mesenteroides.* This supports our result and methodology although the

entire LAB in our study inhibited the growth of *Staphylococcus aureus.* Additionally, *Enterococcus faecium* demonstrated antibacterial activity against *Staphylococcus aureus* (Ammor *et al*. 2006) while Olivares *et al*. (2006) reported antimicrobial activity of *Lactobacillus fermentum* against *Escherichia coli* and *Staphylococcus aureus*.

Therefore, antimicrobial activity of LAB against both Gram positive (*Bacillus cereus* and *Staphylococcus aureus*) and Gram negative bacteria (*Escherichia coli* and *Staphylococcus aureus*) have been corroborated by aforementioned researchers (Ammor *et al*. (2006), Olivares *et al*. (2006), Jara *et al*. (2011), Anyogu *et al*. (2014) and Leite *et al*. (2015).

The antimicrobial activity of unconcentrated and concentrated cell free supernatants of LAB against four pathogenic bacteria was also investigated. The effects of heat, Proteolytic and catalase enzymes on antimicrobial activity of supernatants were also assessed. Concentration of cell free supernatants increased antimicrobial activity of *Lactobacillus pentosus, Lactobacillus plantarum, Lactobacillus coryniformis* and *Streptococcus cristatus, Lactobacillus fermentum, Weissella paramesenteroides, Leuconostoc mesenteroides* subsp. *mesenteroides* and *Weissella confusa*. However, antimicrobial activity of *Lactobacillus fermentum, Weissella paramesenteroides, Leuconostoc mesenteroides* subsp. *mesenteroides* and *Weissella confusa* were affected by heat.

Effects of proteolytic and catalase enzymes on antimicrobial activity of supernatants inhibition of pathogens were variable. Supernatants of *Streptococcus*

cristatus was the most stable, they were not affected by enzymes. Inhibition was seen against all indicator bacteria. Similarly, *Lactobacillus pentosus, Lactobacillus plantarum, Lactobacillus coryniformis* also retained some of their antimicrobial activity. The antimicrobial activity of *Lactobacillus pentosus, Lactobacillus plantarum, Lactobacillus coryniformis* and *Streptococcus cristatus* could be as a result of production of organic acids since no inhibition of pathogens was observed after neutralisation of concentrated cell free supernatants. The antimicrobial activity could also be attributed to production of peptides (bacteriocins) and hydrogen peroxide.

Jara *et al*. (2011) studied inhibitory activity of *Lactobacillus plantarum, Lactobacillus acidophilus, Lactobacillus salivarius, Lactobacillus paracasei, Lactobacillus curvatus* isolated from breast milk on gastrointestinal pathogenic bacteria (*Escherichia coli*, *Salmonella* Enteritidis and *Shigella* ssp.). The agar diffusion assay was used for assessing the antimicrobial activity. The result of the study indicated that all the *Lactobacillus* ssp. inhibited growth of the three pathogenic bacteria but *Lactobacillus plantarum* showed the strongest zone of inhibition against *Salmonella* Enteritidis. Similar trend was also seen in our study.

Additionally, Al-Otaibi *et al*. (2012), studied antimicrobial activity of *Lactobacillus* and *Lactococcus* isolated from Saudi fermented dairy products. In their findings, they stated that about 30% of LAB that show antimicrobial activity against pathogens in agar spot test failed to produce the same antimicrobial activity in the agar well diffusion assay.

In agar spot test, LAB are able to continue to grow and secrete antimicrobial substances but when minimum amount of cell free supernatant is dispensed in agar well diffusion assay, pathogenic bacteria might be able to resist antimicrobial substances present in cell free supernatant. Another factor that could prevent inhibition is poor diffusion and permeation of supernatant through agar.

Al-Otaibi *et al*. (2012) also studied effect of heat treatment (63 ^{o}C for 30 min) on cell free supernatant of *Lactobacillus* and *Lactococcus* against *Staph. aureus*, *Salmonella* species, *E. coli* and *Listeria monocytogenes*. They observed inhibition of pathogens, indicating efficiency of antimicrobial substances despite heat treatment at this temperature and time. A similar trend was also seen in the study herein reported because heat treated concentrated cell free supernatant of *Lactobacillus pentosus, Lactobacillus plantarum, Lactobacillus coryniformis* and *Streptococcus cristatus* inhibited growth of all the four pathogens. According to Al-Otaibi *et al*. (2012), antimicrobial activity of cell free supernatants of *Lactobacillus bulgaricus* and *Lactococcus lactis* were lost when treated with proteinase K and pepsin. Furthermore, antimicrobial activity of *Lactobacillus plantarum* TF711 was inactivated by proteinase K, pronase E, trypsin and pepsin but was active with lipase A enzymes (Hernandez *et al*. 2005). In this study, antimicrobial substances of *Streptococcus cristatus* were not affected by enzyme reaction, however, antimicrobial activity *Lactobacillus pentosus, Lactobacillus plantarum, Lactobacillus coryniformis* was not active against all pathogens.

Anyogu *et al*. (2014) reported that no inhibition was seen in *Escherichia coli, Salmonella typhimurium, Staphylococcus aureus* and *Bacillus cereus* after

neutralisation of cell free supernatants of *Weissella confusa, Lactobacillus plantarum, Leuconostoc mesenteroides* subsp. *mesenteroides, Enterococcus faecium* and *Weissella paramesenteroides*. A similar trend was also seen in the study herein reported.

4.5 Conclusion

The antimicrobial properties of LAB have been attributed to their probiotic potential. The inhibitory effect of the LAB against both Gram positive and Gram negative bacteria was investigated. The LAB has exhibited broad inhibitory spectrum against the indicator (pathogenic) bacteria in the buffered and unbuffered agar spot tests. Some of the LAB also inhibited growth of some of the pathogens in the agar well diffusion assay.

The results herein reported indicate that the LAB studied have the potential to be considered probiotic. The antimicrobial properties of cell free supernatants of some of the LAB studied inhibited pathogenic bacteria. Heat did not affect antimicrobial activity of *Lactobacillus pentosus, Lactobacillus plantarum, Lactobacillus coryniformis* and *Streptococcus cristatus.* Antimicrobial activities of the LAB were also not affected by some enzymes. Moreover, antimicrobial properties of the studied LAB could be attributed to production of lactic acid, hydrogen peroxide and bacteriocins. This study has therefore, contributed to the knowledge of the antimicrobial activity of some lactic acid bacteria that have been identified in some samples of Nigerian human breast milk.

CHAPTER FIVE: CHOLESTEROL ASSIMILATION, BILE SALT HYDROLASE AND EXOPOLYSACCHARIDE PRODUCTION BY LACTIC ACID BACTERIA ISOLATED FROM HUMAN BREAST MILK

5.1 Introduction

Hypercholesterolemia (high blood cholesterol) is an important risk factor for heart disease. Alternative to conventional drugs such as statins (simvastatin and lovastatin) with fewer side effects is on the rise. Some beneficial lactic acid bacteria (LAB) have shown promising cholesterol-lowering ability. Moreover, Anandharaj and Sivasankari, (2014) has reported the cholesterol-reducing property of *Lactobacillus oris* species that was isolated from human breast milk. Amongst beneficial effects of LAB is lowering of serum cholesterol which is evident in many studies in humans, animals and *in vitro*. (Pereira *et al*. 2003, Begley *et al*. 2006, Damodharan *et al*. 2015, Saraniya and Jeevaratnam 2015, Shehata *et al*. 2016 and Yadav *et al*. 2016).

Bile salt hydrolase (BSH) activity of LAB has been attributed to serum cholesterol reduction (Damoddharan *et al*. 2015). Bile salt hydrolase (BSH) is an enzyme that is produced by some LAB which causes the deconjugation of bile salts. Deconjugated bile acids (glycine and taurine) are therefore less soluble and less reabsorbed into blood. This process facilitates excretion of bile acids in feces (Vianna de Souza and Dias, 2017). Ability of LAB to hydrolyse bile salt through production of hydrolase enzymes is a vital criterion in selection of probiotic bacteria (FAO/WHO, 2002). Bile salt hydrolase activity has been exhibited by many LAB species with probiotic potential especially *Lactobacillus* spps. (Damodharan *et al*. 2015 and Yadav *et al*. 2016). Additionally, Shehata *et al*. 2016 have reported the BSH activity and cholesterol assimilation of some *Lactobacillus* species including *Lactobacillus paracasei* and *Lactobacillus*

delbrueckii. Furthermore, Anandharaj and Sivasankari, (2014) and Riaz-Rajoka *et al*. (2017) have reported the BSH activity of *Lactobacillus rhamnosus* and *Lactobacillus oris* species that were isolated from human breast milk. However, BSH activity could interfere with normal lipid digestion because deconjugated bile salts are less efficient in the emulsification of dietary lipids (Begley *et al*. 2006).

Bacterial exopolysaccharides (EPS) are long-chain polysaccharides which could adhere to cell surface or secreted into extracellular environment as loose slime. Some exopolysaccharide are prebiotic that stimulates growth of beneficial bacteria. Moreover, exopolysaccharide producing LAB can efficiently pass to gastrointestinal tract (Patil *et al*. 2015). Additionally, exopolysaccharides are bioactive substances that have beneficial effects on human health (Panthavee *et al*. 2017). Apart from EPS textural quality in dairy fermented foods, EPS secreted by beneficial LAB can reduce obesity, cholesterol, ulcer, gastritis and other inflammatory reactions (Joshi and Koijam, 2014, Patil *et al*. 2015 and Panthavee *et al*. 2017). Moreover, Jiang *et al*. (2016) and Riaz-Rajoka *et al*. (2017) have reported production of EPS by *Lactobacillus plantarum* and *Lactobacillus rhamnosus* species that were isolated from human breast milk.

The aim of the study described in this chapter was to examine cholesterol assimilation, bile salt hydrolase and exopolysaccharide production by some of the identified LAB. Ability of LAB to assimilate cholesterol, produce bile salt hydrolase and exopolysaccharide are some of the functional properties of probiotics. The specific objectives of this study were:

- To evaluate cholesterol reducing potential of the identified LAB.
- To assess bile salt hydrolase activity of the LAB.
- To examine exopolysaccharide production by LAB.

The LAB selected for investigation were:

- *Enterococcus faecium* (A1a[Mc])
- *Lactobacillus coryniformis* (T3b[Mc])
- *Lactobacillus fermentum* (O3[M])
- *Lactobacillus pentosus* (T2a[M])
- *Lactobacillus plantarum* (P3[M])
- *Leuconostoc mesenteroides* subsp. *lactis* (R1[M])
- *Leuconostoc mesenteroides* subsp. *mesenteroides* (C2c[Mc])
- *Streptococcus cristatus* (Q1[M])
- *Streptococcus salivarius* subsp. *thermophilus* (S2b[Mc])
- *Weissella confusa* (M2c[M])
- *Weissella paramesenteroides* (D1[Mc])

Each of the LAB selected represent other identified LAB of the same genus and species. The selection was based on similarity in their rep-PCR profiles.

The control, *Enterococcus casseliflavus* was kindly provided by the London Metropolitan University's Microbiology Unit of Science Laboratory.

5.2 Materials and methods

5.2.1 Inoculum preparation

A loopful of several colonies of a 24 h culture of isolate grown on MRS agar was sub-cultured into 1 ml maximum recovery diluent (MRD) and drops of this was added to 9 ml of MRD in sterile bottles with black caps until a value of 0.5 on the MacFarland standard was achieved, which gave an approximate concentration of 10^8 cfu/ml, this was the inoculum for the experiment.

5.2.2 Exopolysaccharide (EPS) production by lactic acid bacteria

The initial qualitative exopolysaccharide production study was carried out according to the methods of Joshi and Koijam, (2014), Zergui *et al*. (2015) and Angmo *et al*. (2015). An aliquot of 100 µl inoculum was spread on ruthenium red milk agar (10% (w/v) skim milk powder (70166, Fluka, UK) 1% (w/v) sucrose (84100, Sigma, UK), ruthenium red (2751, Sigma, UK) 0.08% (w/v) and plain agar (LP0012, Oxoid, UK) 1.5% (w/v)). Plates were incubated anaerobically at 37 ^{o}C for 48 h. White coloured colonies indicate EPS production. The ruthenium red was filter sterilised with first 0.45 micron then 0.2 micron nalgene syringe filters before adding to molten agar containing skim milk powder and sucrose.

An aliquot of 100 µl inoculum was spread on MRS agar supplemented with 5% (w/v) sucrose (84100, Sigma, UK) and 0.08% (w/v) ruthenium red (2751, Sigma, UK). Also, an aliquot of 100 µl inoculum was spread on MRS agar supplemented with 5% (w/v) sucrose. Plates were incubated anaerobically at 37 ^{o}C for 48 h. White coloured colonies on MRS ruthenium red agar indicate EPS production.

Shiny viscous colonies on MRS sucrose agar also indicate EPS production. MRS ruthenium red agar and MRS sucrose agar were used in order to compare EPS production by LAB in both media.

Exopolysaccharide positive isolates were screened quantitatively for exopolysaccharide production using the methods of Akabanda *et al*. (2014), Joshi and Koijam, (2014) and Larouchi *et al*. (2014). An aliquot of 1 ml inoculum (10^8 cfu/ml) was placed in 5 ml MRS broth containing 5% (w/v) sucrose. The suspension was incubated at 37 ^{o}C for 24 h. Culture was centrifuged at 10,000 x g for 10 min. An aliquot of 1 ml supernatant was placed in 3 ml cold 95% ethanol (652261, Sigma, UK) and kept at 4 ^{o}C for 24 h. The mixture was centrifuged at 10,000 x g for 10 min and 3 ml deionised water was placed in precipitated pellets (EPS sample). Total amount of carbohydrate content in pellet was determined by the phenol-sulphuric acid method. An aliquot of 1 ml EPS sample was mixed with 1 ml deionised water. An aliquot of 1 ml 6% (v/v) phenol aqueous solution (P02632, Sigma, UK) was added to the mixture followed by rapid addition of 5ml of 95% (v/v) sulphuric acid (435589, Sigma, UK). The mixture was vigorously mixed and was incubated at room temperature for 20 min. An aliquot of 500 µl of each sample was diluted with 500 µl deionised water. Absorbance was measured at 490 nm using spectrophotometer (Jenway, 7315). MRS broth (69966, Sigma, UK) containing 5% (w/v) sucrose was used as control. Experiments were carried out in duplicate. The concentration of EPS was calculated using the Beer Lambert's law equation following the method of Indimuli *et al*. (2015) and Khamis *et al*. (2017). The Beer Lambert's law equation is shown below;

$A = \varepsilon LC$

Where A = amount of light absorb by sample at a given wavelength

ε = molar absorptivity

L = distance that light travels through solution (cuvette path length)

C = concentration of absorbing sample per volume

5.2.3 Bile salt hydrolase activity

The bile salt hydrolase activity study was carried out according to the methods of Saraniya and Jeevaratnam (2015), Shehata *et al*. (2016) and Yadav *et al*. (2016). An aliquot of 100 µl inoculum was spread on MRS agar (69964, Sigma, UK) plates supplemented with 0.37g/L (0.037% (w/v) calcium chloride (449709, Sigma, UK) and 5g/L (0.5% (w/v)) sodium salt of taurodeoxycholic acid (TDCA, T0557, Sigma, UK). Unsupplemented MRS agar plates were used as control. The plates were incubated anaerobically at 37 ^{o}C for 48 h. White precipitated colonies on MRS (TDCA) agar plates indicate deconjugation of bile salt.

5.2.4 Cholesterol assimilation

The cholesterol assimilation study was carried out according to the methods of Iranmanesh *et al*. (2014), Tomaro-Duchesneau *et al*. (2014), Saraniya and Jeevaratnam (2015), Shehata *et al*. (2016) and Yadav *et al*. (2016).

Filter sterilised water soluble cholesterol (10mg/100ml, C1145, Sigma, UK) was added to MRS broth supplemented with 0.3g/100ml ox-bile salt (70168, Fluka, UK). The mixture was inoculated with 1% each of LAB culture and incubated

anaerobically at 37 ^{o}C for 24 h. Uninoculated mixture (cholesterol and MRS broth supplemented with 0.3g/100ml bile salt) was kept as control. After incubation, cultured broth was centrifuged at 10,000 x g for 10 min. An aliquot of 1 ml of supernatant was placed in 1 ml potassium hydroxide (33%w/v, 30614, Sigma, UK) and 2 ml absolute ethanol (46139, Sigma, UK) was added. The mixture was placed in water bath at 37 ^{o}C for 15 min. After cooling to 22 ^{o}C, 2 ml deionised water and 3 ml hexane (296090, Sigma, UK) was added and mixed thoroughly for one minute. This was kept at room temperature for 15 min to allow phase separation. An aliquot of 1 ml hexane layer was placed in a tube and the tube was placed in water bath at 65 ^{o}C to allow evaporation of solvent. Once dried, 2 ml o-phthalaldehyde reagent (P0632, Sigma, UK) was added and mixed thoroughly for one minute. After mixing, an aliquot of 0.5 ml concentrated sulphuric acid (339741, Sigma, UK) was added and mixed thoroughly for one minute. The mixture was kept at room temperature for 10 min. Absorbance of inoculated broth sample and uninoculated broth sample was read at 550 nm using a spectrophotometer. Ability of LAB to remove cholesterol from broth was calculated using the formula below;

A = (B – C/B) X 100 (Saraniya and Jeevaratnam 2015 and Yadav *et al*. 2016)

Where A = % cholesterol removed

B = Absorbance of uninoculated MRS/cholesterol broth sample

C = Absorbance of inoculated MRS/cholesterol broth sample

Experiments were carried out in duplicate.

5.3 Results

5.3.1 Exopolysaccharide (EPS) production and bile salt hydrolase activity

The ability of the eleven selected LAB to produce exopolysaccharide, deconjugate bile salt and reduce cholesterol was examined. All 11 LAB produced exopolysaccharides on MRS ruthenium red agar and MRS sucrose during the initial qualitative screening (Table 5.1 and Figure 5.1 to 5.6). But on ruthenium red milk agar, *Lactobacillus coryniformis, Lactobacillus fermentum* and *Weissella paramesenteroides* did not produce exopolysaccharides. The entire 11 LAB produced EPS at a concentration of 0.452 to 0.542 mg/ml during the quantitative evaluation. *Lactobacillus pentosus* had the highest EPS yield of 0.542 mg/ml which is higher than the bacterium that was used as control (*Enterococcus casseliflavus*). The EPS yield of *Enterococcus casseliflavus* was 0.510 mg/ml (Table 5.2 and Figure 5.7). Similarly, the entire 11 LAB deconjugated bile salt of taurodeoxycholic acid (Table 5.1 and Figure 5.8 to 5.9).

Table 5.1 Exopolysaccharide (EPS) production by lactic acid bacteria and bile salt hydrolase activity.

LAB	RRM	MRSRR	MRSS	MRSTDCA
Leuconostoc mesenteroides subsp. *mesenteroides*	+	+	+	+
Weissella paramesenteroides	-	+	+	+
Lactobacillus fermentum	-	+	+	+
Lactobacillus pentosus	+	+	+	+
Weissella confusa	+	+	+	+
Streptococcus salivarius subsp. *thermophilus*	+	+	+	+
Enterococcus faecium	+	+	+	+
Lactobacillus plantarum	+	+	+	+
Leuconostoc mesenteroides subsp. *lactis*	+	+	+	+
Lactobacillus coryniformis	-	+	+	+
Streptococcus cristatus	+	+	+	+

RRM = Ruthenium red milk agar

MRSRR = MRS ruthenium red agar

MRSS = MRS sucrose agar

MRSTDCA = MRS taurodeoxycholic acid

(-) = no EPS production

(+) = EPS production or deconjugation of bile salt

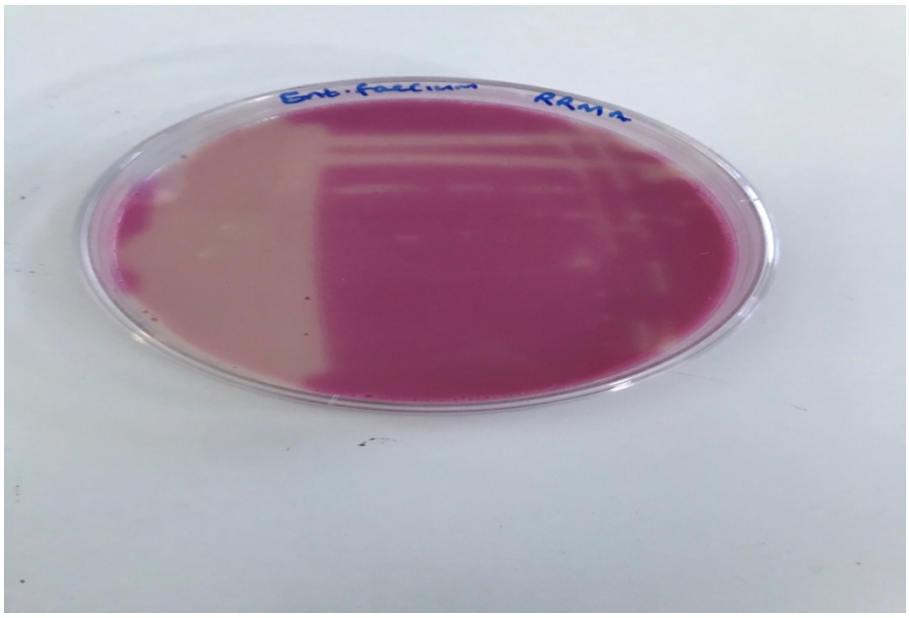

Figure 5.1: Exopolysaccharide production by *Enterococcus faecium* on ruthenium red milk agar

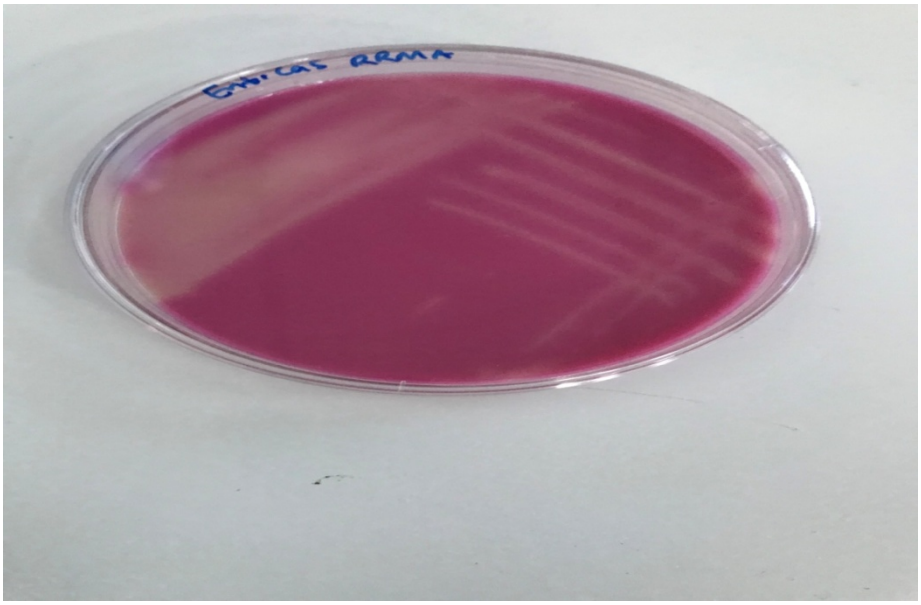

Figure 5.2: Exopolysaccharide production by *Enterococcus casseliflavus* (control) on ruthenium red milk agar

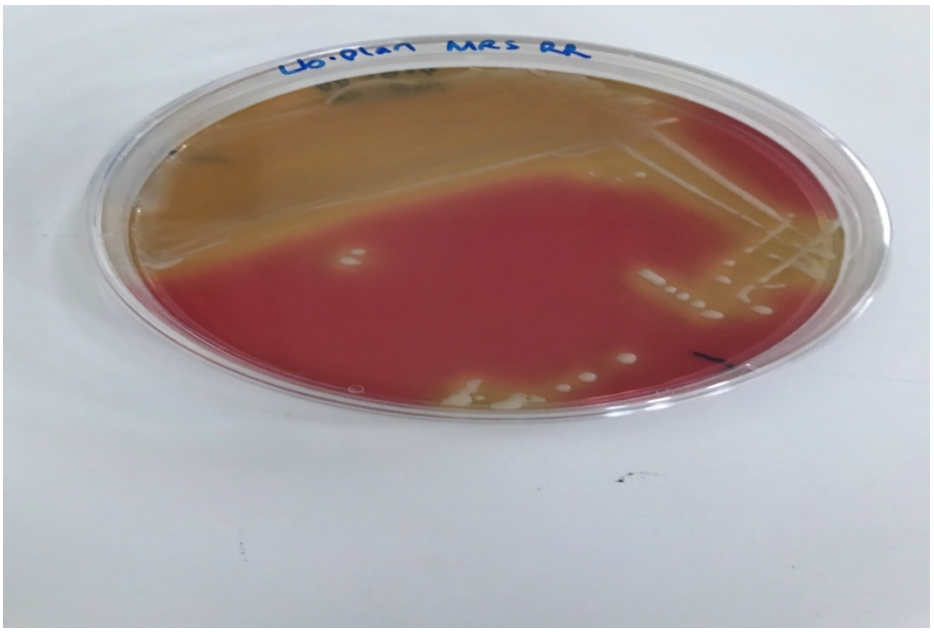

Figure 5.3: Exopolysaccharide production by *Lactobacillus plantarum* on MRS ruthenium agar

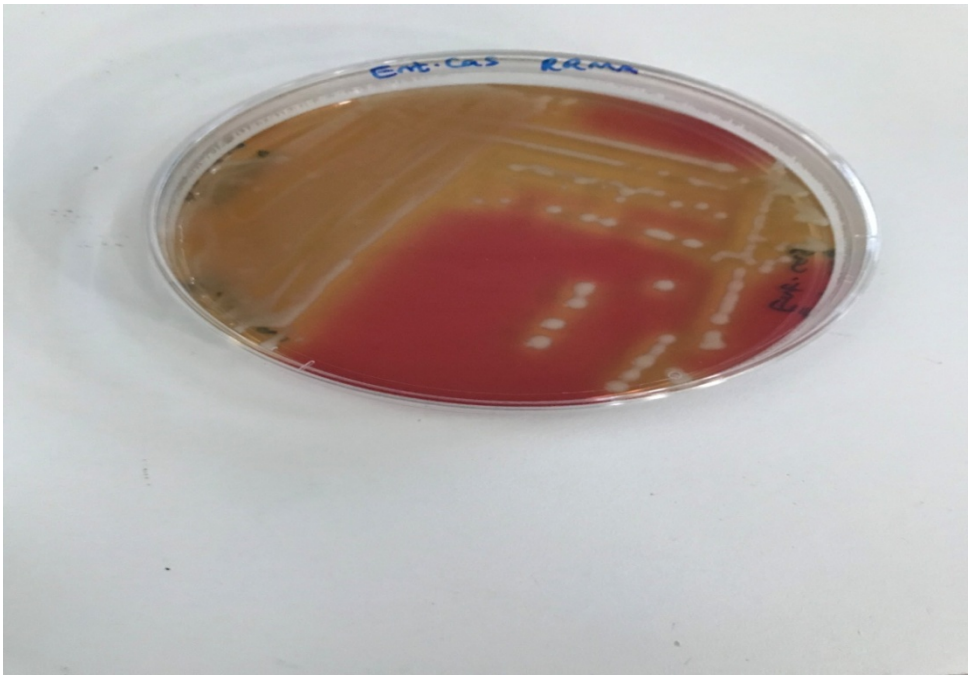

Figure 5.4: Exopolysaccharide production by *Enterococcus casseliflavus* (control) on MRS ruthenium agar

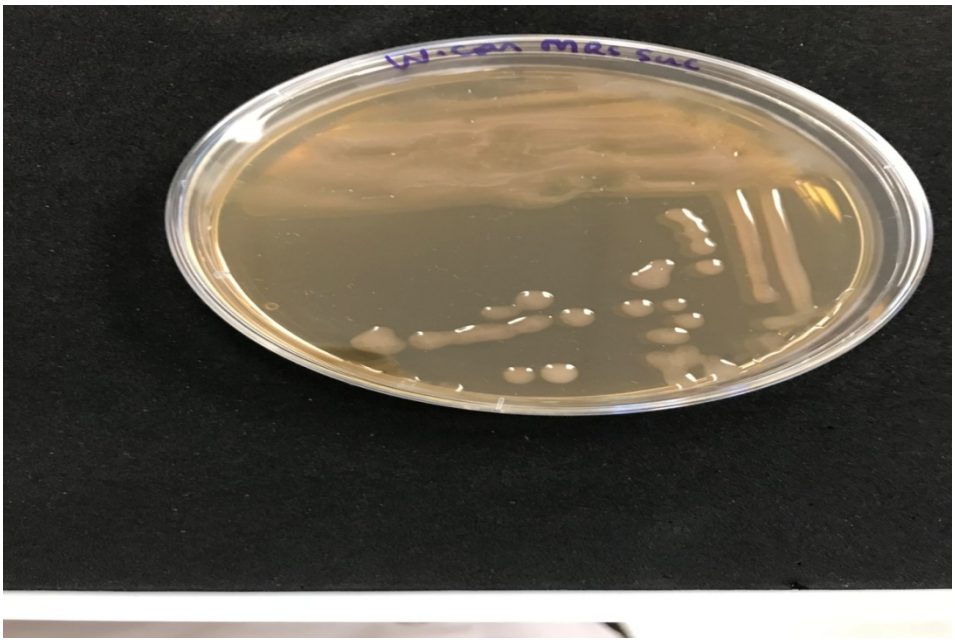

Figure 5.5: Exopolysaccharide production by *Weissella confusa* on MRS sucrose agar

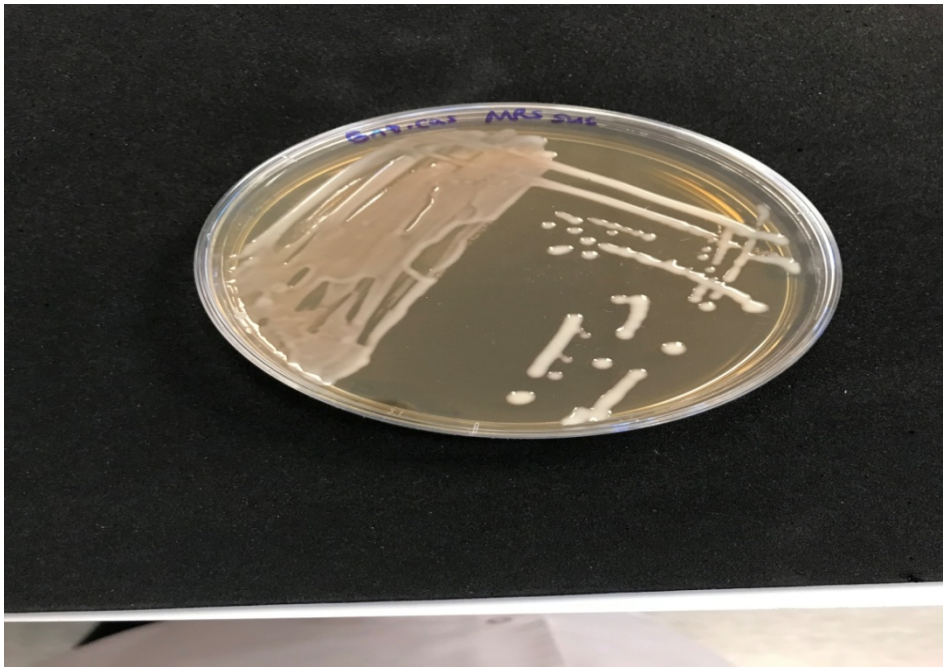

Figure 5.6: Exopolysaccharide production by *Enterococcus casseliflavus* (control) on MRS sucrose agar

Table 5.2 Absorbance and concentration of EPS produced by lactic acid bacteria

LAB	Absorbance (490 nm)	Concentration of EPS (mg/ml)
Leuconostoc mesenteroides subsp. *mesenteroides*	0.851 ± 0.01	0.473 ± 0.01
Weissella paramesenteroides	0.883 ± 0.03	0.491 ± 0.02
Lactobacillus fermentum	0.852 ± 0.01	0.473 ± 0.01
Lactobacillus pentosus	0.975 ± 0.02	0.542 ± 0.02
Weissella confusa	0.957 ± 0.04	0.532 ± 0.03
Streptococcus salivarius subsp. *thermophilus*	0.880 ± 0.02	0.489 ± 0.02
Enterococcus faecium	0.943 ± 0.02	0.521 ± 0.01
Lactobacillus plantarum	0.813 ± 0.01	0.452 ± 0.01
Leuconostoc mesenteroides subsp. *lactis*	0.908 ± 0.03	0.505 ± 0.01
Lactobacillus coryniformis	0.853 ± 0.05	0.474 ± 0.03
Streptococcus cristatus	0.966 ± 0.01	0.537 ± 0.01
Enterococcus casseliflavus	0.918 ± 0.03	0.510 ± 0.02

Data are mean ± standard deviation of duplicate experiments.

The concentration of EPS yield was calculated using the Beer Lambert's law equation;

$A = \varepsilon LC$

Where A = amount of light absorbed by sample at a given wavelength

ε = molar absorptivity = 0.899

L = distance that light travels through solution (cuvette path length) = 1cm

C = concentration of absorbing sample per volume

Initial concentration of samples were diluted 2 fold (500µl:500µl) = 1/2

Therefore, calculated concentration (C) was multiplied by 0.5

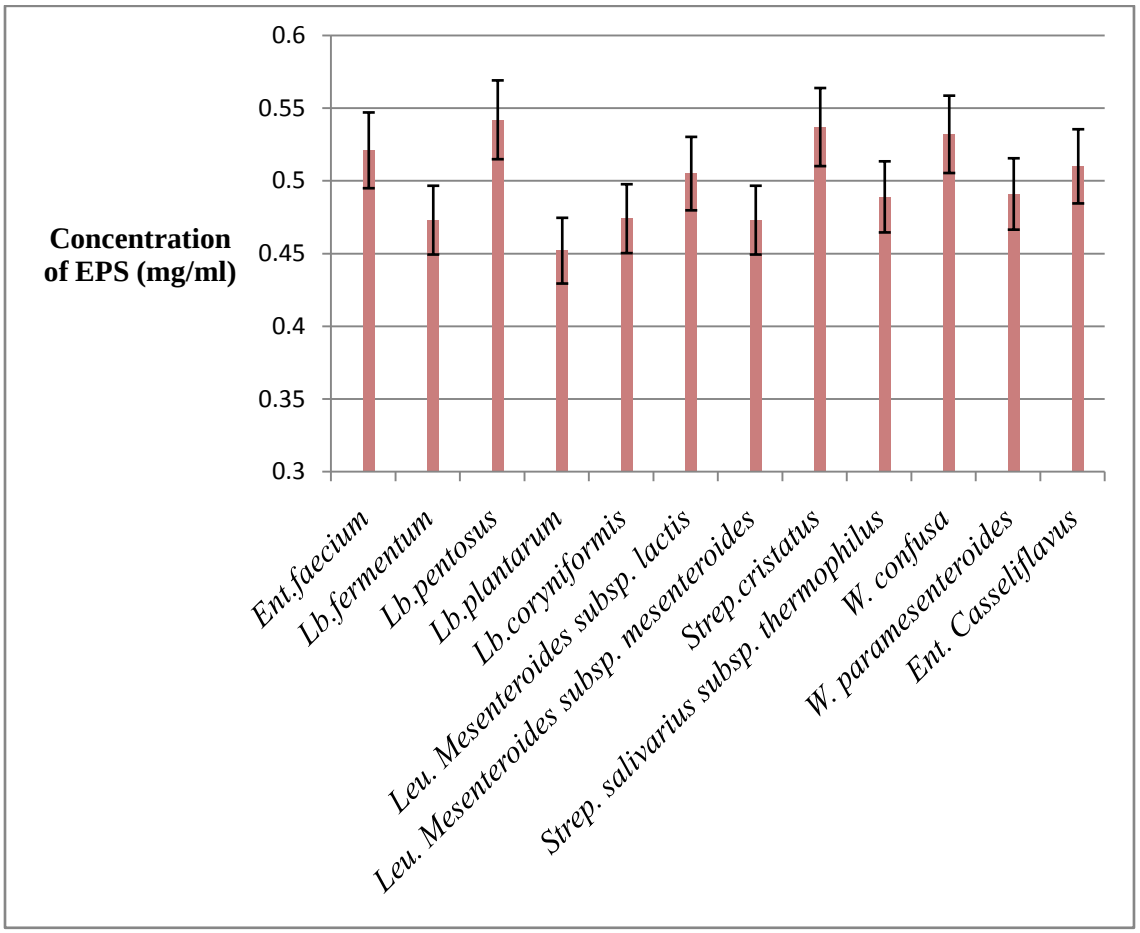

Figure 5.7: The concentration of EPS produced by LAB isolated from human breast milk

The error bars are standard deviation of duplicate experiments.

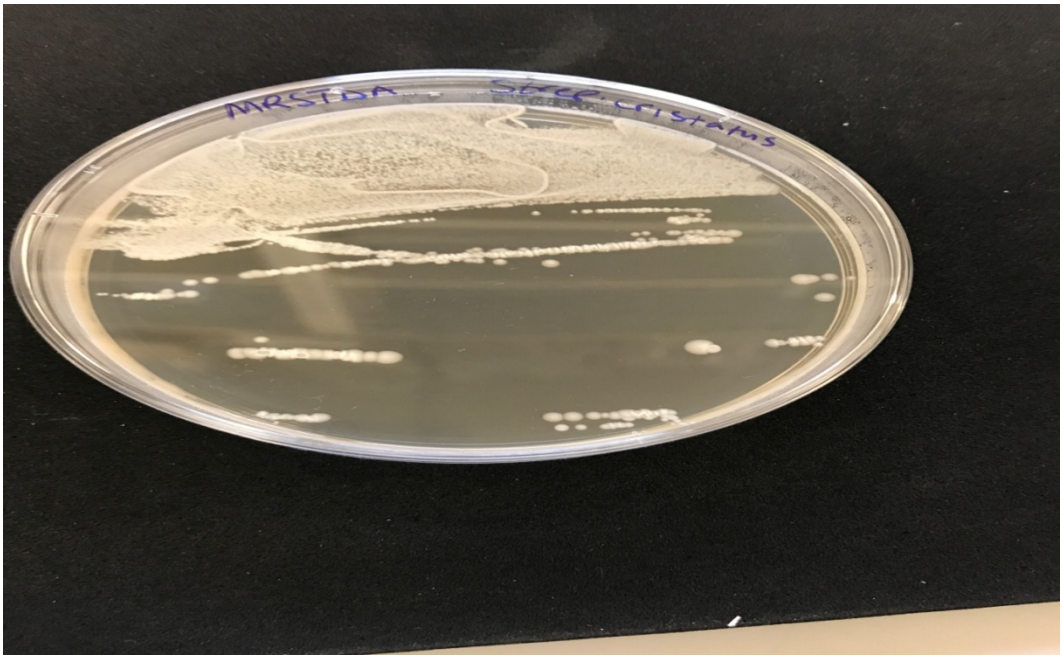

Figure 5.8: Deconjugation of bile salt by *Streptococcus cristatus* on MRS taurodeoxycholic acid agar

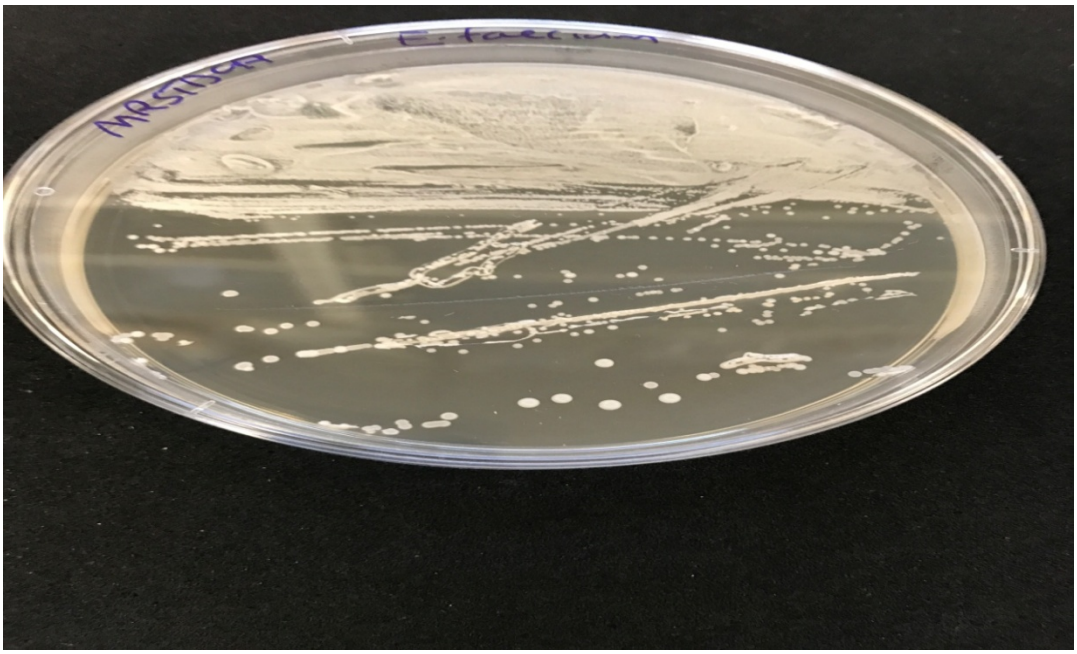

Figure 5.9: Deconjugation of bile salt by *Enterococcus faecium* on MRS taurodeoxycholic acid agar

5.3.2 Cholesterol assimilation

Cholesterol assimilation potential of LAB was evaluated *in vitro* in the presence of ox- bile. The entire 11LAB exhibited cholesterol reducing ability, the highest cholesterol assimilation was observed in *Lactobacillus plantarum* with 68% reduction. Followed by *Lactobacillus fermentum* and the lowest assimilation percentage was observed in *Weissella confusa* with 46±8.48%. (Table 5.3 and Figure 5.10).

Table 5.3 Cholesterol assimilation by lactic acid bacteria isolated from human breast milk

LAB	Absorbance (550 nm)	Cholesterol assimilated (%)
Leuconostoc mesenteroides subsp. *mesenteroides*	0.136 ± 0.02	59 ± 8.48
Weissella paramesenteroides	0.142 ± 0.03	57 ± 12.72
Lactobacillus fermentum	0.118 ± 0.01	65 ± 4.24
Lactobacillus pentosus	0.133 ± 0.03	60 ± 12.72
Weissella confusa	0.181 ± 0.02	46 ± 8.48
Streptococcus salivarius subsp. *thermophilus*	0.125 ± 0.01	62 ± 4.24
Enterococcus faecium	0.159 ± 0.05	52 ± 21.21
Lactobacillus plantarum	0.107 ± 0.00	68 ± 0.00
Leuconostoc mesenteroides subsp. *lactis*	0.120 ± 0.02	64 ± 8.48
Lactobacillus coryniformis	0.122 ± 0.01	63 ± 4.24
Streptococcus cristatus	0.130 ± 0.02	61 ± 8.48

Data are mean ± standard deviation of duplicate experiments.

A = (B – C/B) X 100 (Saraniya and Jeevaratnam 2015 and Yadav *et al*. 2016)

Where A = % cholesterol removed

B = Absorbance of control (uninoculated MRSbile + cholesterol (10mg/100ml) = 0.334

C = Absorbance value of each LAB inoculated into MRS/cholesterol broth sample

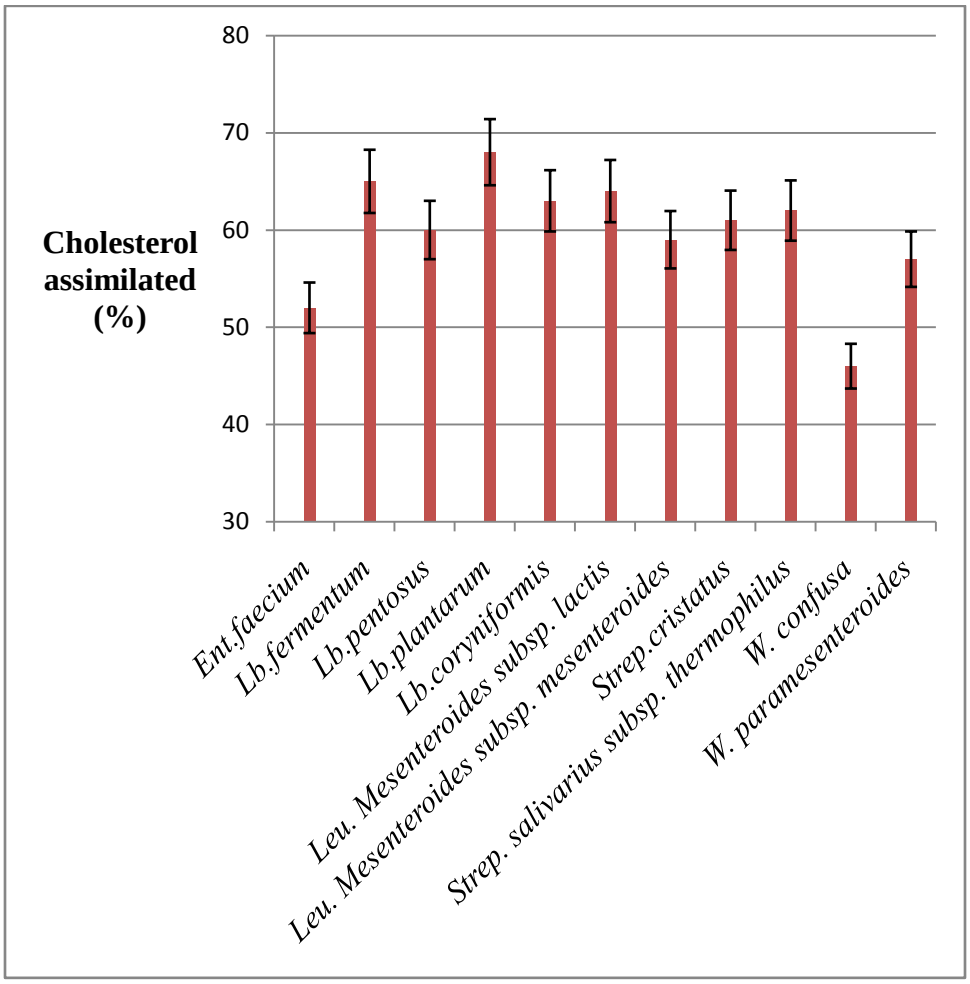

Figure 5.10: Cholesterol assimilation by LAB isolated from human breast milk

The error bars are standard deviation of duplicate experiments.

5.4 Discussion

Exopolysaccharide production (EPS) and bile salt hydrolase activity of LAB are important requirement in the selection of probiotic LAB. Similarly cholesterol assimilation potential of LAB is also an essential attribute of probiotic LAB. The Exopolysaccharide production (EPS), bile salt hydrolase activity and cholesterol assimilation by lactic acid bacteria was therefore investigated in this study. During the qualitative screening of EPS, all the LAB were able to produce EPS on MRS ruthenium red agar and MRS sucrose agar except for ruthenium red milk agar on which *Lactobacillus coryniformis, Lactobacillus fermentum* and *Weissella paramesenteroides* did not produce exopolysaccharides. For the quantitative analysis of EPS, the entire LAB produced EPS at a concentration of 0.452 to 0.542 mg/ml. *Lactobacillus pentosus* produced the highest concentration of EPS of 0.542 (mg/ml) while *Lactobacillus plantarum* produced the lowest. Total amount of EPS produced by LAB is greatly influenced by type of sugars present in growth medium (Hongpattarakere *et al*. 2012 and Joshi and Koijan 2014). The ability of the entire LAB in this study to produce EPS on MRS ruthenium red agar and MRS sucrose agar could be attributed to presences of high concentration of two kinds of sugars in the medium. MRS agar already has glucose and was further supplemented with 5% sucrose. Furthermore, MRS is an excellent medium for growth of LAB. Similarly, the MRS broth used for the quantitative determination of EPS was also supplemented with 5% sucrose. The amount of EPS produced from each LAB in the culture broth supports the agar screening on the MRS agar. The negative EPS production of *Lactobacillus coryniformis, Lactobacillus fermentum* and *Weissella paramesenteroides* on ruthenium red milk

agar could be attributed to low concentration of 1% sucrose and this sugar was the sole carbon source. EPS production on MRS medium supplemented with high concentration of sucrose has been shown to be optimum (Van Geel Schutten *et al*. 1998, Hongpattarakere *et al*. 2012 and Joshi and Koijan 2014). The authors also pointed out that sucrose is a very good substrate for ample EPS production, they therefore suggest supplementation of agar and broth for screening of EPS producing LAB with high concentration of this sugar. Jiang *et al*. (2016) reported EPS yield of 426.73 mg/L by *Lactobacillus plantarum* isolated from human breast milk which is higher than the yield obtained for this bacterium (0.452 mg/ml) in this study. However, Sasikumar *et al*. 2017 reported a much higher yield of 2.8 g/L from *Lactobacillus plantarum* that was isolated from jackfruit. Moreover, Riaz-Rajoka *et al*. (2018) reported EPS yield of 461-737.3 mg/L from strains of *Lactobacillus rhamnosus* isolated from human breast milk. Furthermore, Tulumoglu *et al*. (2013) investigated EPS production by some lactobacilli species isolated from children feces, their research findings indicate EPS yield of 70-290 mg/L with *Lactobacillus pentosus* having the highest yield. Similarly, in this research, *Lactobacillus pentosus* had the highest EPS yield. However, Akanbanda *et al*. (2014) reported EPS production of 50-150µg/ml from *Lactobacillus fermentum, Lactobacillus plantarum, Leuconostoc mesenteroides* subsp. *mesenteroides* and *Enterococcus faecium* from fermented milk product. The EPS produced by similar LAB in this study were much higher than that reported by Akanbanda *et al*. (2014). Additionally, Joshi and Koijan (2014) reported an EPS yield of 340.82 mg/L from *Leuconostoc mesenteroides* subsp. *lactis* isolated from

fermented beverage. EPS production is variable among genus and species of LAB, therefore, yields and properties are dependent on kind of strain and species (Riaz-Rajoka *et al*. 2018). EPS polymers do not only act as carbon or energy reservoirs, they are importantly involved in protection of LAB against unfavourable harsh environmental conditions (Salazar *et al*. 2016). In fact, EPS production by LAB and bifidobacteria promote acid and bile resistance and adherence to intestinal mucosa thereby prolonging existence of LAB in gastro intestinal tract (Alp and Aslim, 2010 and Zergui *et al*. 2014). Furthermore, production of EPS by LAB also facilitates their translocation from maternal gut to mammary gland (Jeurink *et al*. 2013). EPS producing LAB play a significant role in colonisation of GIT tract thus, compete favourably with pathogenic bacteria thereby reducing colonisation of pathogens ((Jeurink *et al*. 2013 and Riaz-Rajoka *et al*. 2018). In fact, EPS have been attributed with antioxidant activity, antitumor and cholesterol reducing potential (Joshi and Koijan 2014, Sasikumar *et al*. 2017 and Riaz-Rajoka *et al*. 2018). Moreover, EPS producing LAB are able to bind free bile acids thereby reducing cholesterol level in the GIT (Sasikumar *et al*. 2017).

Cholesterol reduction ability of LAB was assessed *in vitro*, all LAB demonstrated cholesterol lowering ability ranging from 46±8.48 to 68±0.00%. Many strains of lactobacilli and other LAB have been shown to assimilate cholesterol *in vitro* (Tulumoglu *et al*. 2013, Annadraj and Sivasankar 2014, Iranmanesh *et al*. 2014, Saraniya and Jeevaratnam 2015, Sasikumar *et al*. 2017 and Gunyatki and Asan-Ozusaglam 2018). Moreover, Annadraj and Sivasankar (2014) reported 61% assimilation of cholesterol by *Lactobacillus oris* isolated from human breast milk.

This is in agreement with this study as all the *Lactobacillus* species have demonstrated cholesterol assimilation percentage of 60% and above. Similarly, Gunyatki and Asan-Ozusaglam (2018) reported 23.67 to 88.94% cholesterol reducing ability of *Lactobacillus gasseri* strains isolated from human breast milk. Additionally, Tulumoglu *et al*. (2013) also reported 32.4 to 76.5% cholesterol removal by *Lactobacillus pentosus*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus* and *Lactobacillus paracasei* isolated from children feces. Their result indicated highest cholesterol assimilation of 76.5% by *Lactobacillus pentosus* which is much higher than herein reported for *Lactobacillus pentosus* (60%). Furthermore, Saraniya and Jeevaratnam (2015) also indicated 73% cholesterol assimilation by *Lactobacillus pentosus* and 71% by *Lactobacillus plantarum* isolated from fermented soy milk. This is in contrast with this study as similar LAB demonstrated less than 70% cholesterol reduction. But Iranmanesh *et al*. (2014) reported 50% cholesterol assimilation by *Lactobacillus pentosus* and 30% by *Leuconostoc lactis* isolated from traditional Iranian dairy product. This is much lower than the cholesterol assimilation herein reported for similar LAB. Hypercholesterolemia (high blood cholesterol level) is an important risk factor for development of heart disease especially coronary heart disorder (atherosclerosis). Bacteriotherapy is a promising alternative for lowering serum cholesterol (Shehata *et al*. 2016). Some of the proposed mechanisms for cholesterol assimilation by LAB include; deconjugation of bile salts, cholesterol conversion to coprostanol (Coprosterol), production of short-chain fatty acids (SCFAs) and uptake of cholesterol into bacterial cell membrane. (Tsai *et al*. 2014, Tomaro-

Duchesneau *et al*. 2014 and Shehata *et al*. 2016). Cholesterol assimilated into bacterial cells would prevent absorption of this substance from host GIT into blood stream (Zergui *et al*. 2014). These mechanisms have been suggested to facilitate cholesterol metabolism by LAB.

Ability of LAB to produce bile salt hydrolase (BSH) enzyme enhance their tolerance to bile salts thus, minimising serum cholesterol (Shehata *et al*. 2016). In this study all LAB demonstrated ability to produce BSH by deconjugation of bile salt of taurodeoxycholic acid. Shehata *et al*. (2016) indicated BSH activity of *Lactobacillus rhamnosus, Lactobacillus paracasei, Lactobacillus gasseri and Lactococcus lactis* isolated from raw milk and fermented foods. Similarly, Saraniya and Jeevaratnam (2015) also indicated BSH activity of *Lactobacillus pentosus* and *Lactobacillus plantarum* isolated from fermented soy milk. Bile salts are produced from cholesterol in the liver, they become conjugated with glycine or taurine to become bile salts. However, BSH producing LAB deconjugate the bile salts to in oder to detoxify them (Begley *et al*. 2006, Saraniya and Jeevaratnam 2015 and Shehata *et al*. 2016). Moreover, BSH enzyme hydrolyses bile salts to release free primary bile acids (Shehata *et al*. 2016).

EPS, cholesterol assimilation and BSH activity are functional properties used for the characterisation of potential beneficial LAB. Many beneficial LAB have been found to produce EPS and BSH which aid in lowering serum cholesterol (Shehata *et al*. (2016), Sasikumar *et al*. (2017) and Riaz-Rajoka *et al*. (2018).

5.5 Conclusion

The Exopolysaccharide production, bile salt hydrolase activity and cholesterol assimilation by LAB have been attributed to their probiotic potential. The results reported in this study show the probiotic attributes of the LAB studied. The LAB were able to produce EPS, BSH and assimilate cholesterol. EPS production by LAB could be increased by supplementation of MRS medium with high concentration of sucrose. Bile salt hydrolase activity of LAB is linked with potential reduction of cholesterol by LAB *in vitro*. This study has therefore, contributed to the knowledge of the functional properties of some lactic acid bacteria that have been identified in some samples of Nigerian human breast milk.

CHAPTER SIX: ANTIMICROBIAL RESISTANCE OF LACTIC ACID BACTERIA AND DETERMINATION OF RESISTANCE GENES

6.1 Introduction

Antimicrobial resistance (AMR) is a global health issue that needs to be addressed from different perspectives. Antimicrobial resistance is the ability of microbes to survive and proliferate when exposed to antimicrobials that they were originally susceptible to. Antimicrobials are extensively misused and overused in humans, food producing animals, agriculture and aquaculture and this undoubtedly contribute to AMR occurrence (Egervin *et al*. 2009, Jimenez *et al*. 2013, Munoz-Atienza *et al*. 2013, WHO, 2014, FSAI, 2015 and Reis *et al*. 2016). Some antimicrobials used in humans and animals have been in existence for more than 70 years. Antimicrobial resistance to some of the first, second and third generation antibiotics is on the rise, particularly to cephalosporins, carbapenemis, quinolones/fluroquinolones, sulphonomides and tetracycline (FSAI, 2015).

Furthermore, Resistance to antimicrobials can be intrinsic or acquired (Karapetkov *et al*. 2011). Intrinsic resistance is a natural AMR trait exhibited by some LAB. It can be defined as the non-susceptibility of a bacterium to a known concentration of antimicrobial that should be lethal at the appropriate dose. This could be due to permeability barrier and active efflux (Sharma *et al*. 2017). Intrinsic resistance is usually not transferable and does not compromise the safety of LAB (Sharma *et al*. 2017). Resistance of bacteria such as some species of lactic acid bacteria (LAB) to aminoglycosides antibiotics (gentamycin, kanamycin, neomycin and streptomycin) is intrinsic because it is associated with the absence of cytochrome-mediated electron transport that mediates the uptake of antimicrobials (Hummel *et al*. 2007). LAB with intrinsic resistance can survive

high dose of antimicrobials making the bacteria less susceptible to antimicrobials (Rolain, 2013). A bacterium can acquire resistance to antimicrobial substances by receiving new traits through mutations in inherent genes or getting resistance genes through horizontal gene transfer (Florez *et al*. 2016). Horizontal transfer of AMR genes is usually mediated by mobile genetic elements such as plasmids and transposons. LAB with antimicrobial resistance genes exist in nature. They can be found in humans, animals, breast milk and other foods (Ouoba *et al*., 2008; Rolain, 2013, Reis *et al*. 2016, Sharma *et al*. 2017). Moreover, the food chain and gastro intestinal tract are suggested to be important routes for spread of antimicrobial resistant LAB (Egervin *et al*. 2009, Reis *et al*. 2016). Thus, these bacteria can act as vectors for spreading AMR genes from food to humans and/or animals (Ouoba *et al*. 2008, Karapetkov *et al*. 2011, FSAI, 2015).

Some LAB are considered beneficial bacteria and thus referred to as probiotics. But safety of these bacteria may be compromised if they act as vectors for transmission of AMR genes to potential pathogenic bacteria (Ouoba *et al*. 2008 and Sharma *et al*. 2017).

Women of childbearing age are frequently misusing antibiotics in developing world including Nigeria where adequate regulations on antimicrobial substances are ineffective (Oloyemi *et al*. 2010 and Sapkota *et al*. 2010). LAB possessing AMR genes could be transferred vertically from mother to infant, from mother's gastro intestinal tract, during delivery or breast milk feeding (Egervin *et al*. 2009, Rolain, 2013, Kozak *et al*. 2015 and Reis *et al*. 2016).

The aim of the study described in this chapter was to evaluate the AMR profile of LAB isolated from human breast milk and to investigate presence of AMR genes in the bacteria. The screening of LAB for AMR and AMR genes is vital in ensuring the safety of potential probiotic LAB. The specific objectives of this study were:

- To assess the phenotypic AMR profiles of some identified LAB by screening their susceptibility to various antimicrobials.
- To investigate the genetic background of phenotypic resistance by screening for AMR genes in the LAB.

6.2 Materials and methods

The susceptibility of LAB was carried out using the methods of Ouoba *et al* (2008).

The LAB selected for investigation were:

- *Enterococcus faecium* (A1a[Mc])
- *Lactobacillus coryniformis* (T3b[Mc])
- *Lactobacillus fermentum* (O3[M])
- *Lactobacillus pentosus* (T2a[M])
- *Lactobacillus plantarum* (P3[M])
- *Leuconostoc mesenteroides* subsp. *lactis* (R1[M])
- *Leuconostoc mesenteroides* subsp. *mesenteroides* (C2c[Mc])
- *Streptococcus cristatus* (Q1[M])

- *Streptococcus salivarius* subsp. *thermophilus* (S2b[Mc])
- *Weissella confusa* (M2c[M])
- *Weissella paramesenteroides* (D1[Mc])

Each of the LAB selected represent other identified LAB of the same genus and species. The selection was based on similarity in their rep-PCR profiles.

6.2.1 Positive control bacteria

Positive control bacteria were used for the AMR gene study and are shown in Table 6.1. The positive controls were kindly provided by the European Union Reference Laboratory for AMR, National Food Institute, Technical University of Denmark (DTU, Lyngby, Denmark).

Table 6.1 Positive control bacteria used for the detection of AMR genes

Bacteria	Gene
Escherichia coli K13	*aac*(3″) IV
Salmonella tyhpimurium DT104	*aad*A
Enterococcus faecalis PEF4	*aad*E
No positive control	*aac*(6″)*aph*(2″)
Escherichia coli K12	*ant*(2″)-1
Staphylococcus aureus E19771	*blaz*A
Staphylococcus aureus Tn554	*erm*(A)
Enterococcus faecalis JH2-2	*erm*(B)
Staphylococcus aureus PSE55	*erm*(C)
Staphylococcus aureus 50A247	*mec*A
Staphylococcus aureus LGA251	*mec*C
Escherichia coli RSF1010	*str*A
Escherichia coli RSF1010	*str*B
Staphylococusintermedius 2567	*tet*(K)
Staphylococcus aureus PSTS9	*tet*(L)
Salmonella intermedius 2567	*tet*(M)
Camplylobacter coli P1P1433	*tet*(O)
Escherichia coli	*tet*(Q)
Listeria monocytogenes BM4210/PIP811	*tet*(S)
Escherichia coli	*tet*(W)
Enterococcus faecium BH4147	*van*A
Enterococcus faecalis V583	*van*B
Enterococcus faecium 6605	*van*X

6.2.2 Assessment of the susceptibility of lactic acid bacteria to antimicrobials

Antimicrobial susceptibility of LAB was evaluated using Gram positive minimal inhibitory concentration (MIC) plates for eighteen antimicrobials (GPN3F, Trek diagnostic systems, Thermo Scientific, UK) as shown in (Table 6.2a and 6.2b). The MIC was determined by the broth microdilution method according to the method of Ouoba *et al*. (2008).

6.2.2.1 Inoculum preparation

A loopful of colonies of 48 h culture of isolates grown on MRS agar was placed in 1ml sterile distilled water. The suspension was centrifuged for 1 min at 13,000 x g (Eppendorf 5415R, UK), the supernatant discarded and the pellet dissolved in 500 µl maximum recovery diluent (MRD). Drops of the suspension were added to 10 ml MRD in sterile screw capped tubes until a value of 0.5 MacFarland standard (10^8cfu/ml) was achieved using nephelometer (Sensititre, SN437R03N007 Trek diagnostic systems, UK).

6.2.2.2 Determination of the MIC

An aliquot of 100 µl of the 0.5 Macfarland standard suspension was added into 20 ml MRS broth and transferred into a sterile petri dish. An aliquot of 50 µl of suspension was inoculated into each well of the microtitre plates that contain the different antimicrobials. Each microtitre plate was covered with a plastic film and incubated anaerobically at 37 ^{o}C for 48 h. A purity check was performed by streaking a loopful of the remaining suspension on MRS agar plates. After the incubation time, the MIC plates were observed with a magnifying sensititre manual mirror. Visible growth (precipitated cells) or no visible growth were recorded on the GPN3F sensititre Gram positive plate format sheet.

To determine the susceptibility profile of the LAB, breakpoints for the antimicrobials proposed by the European Food Safety Agency (EFSA), the European Committee on Antimicrobial Susceptibility testing (EUCAST), the Clinical and Laboratory Standards Institute (CLSI) and other published literatures

(Table 6.2a and 6.2b) were used. Breakpoint is a known concentration of antimicrobial that indicates whether a bacterium is susceptible or resistance to the antimicrobial. The MIC obtained for each antimicrobial was compared with the proposed breakpoint and the susceptibility determined. Each bacterium was considered resistant if the MIC was greater than the established breakpoint and susceptible if less than the breakpoint.

Table 6.2a Proposed Breakpoints for the LAB studied

Antimicrobials/ concentration range	LAB/Breakpoints (µg/ml)				
	Lb plantarum	*Lb. pentosus*	*Lb. fermentum*	*Lb. coryniformis*	*Leu. mesenteroides*
Ampicillin (0.12-16)	2[a]	2[a]	2[a]	2[a]*	2[a]
Ceftriaxone (8-64)	4*	4*	4*	4*	4*
Ciprofloxacin (0.5-2)	4[d]	4*	4[c]	4*	>32[e]
Clindamycin (0.12-1)	2[a]	2[a]	1[a]	1[a]	1[a]
Daptomycin (0.25-8)	4[f]	4[f]	4[f]	4[f]	4*
Erythromycin (0.25-4)	1[a]	1[a]	1[a]	1[a]	1[a]
Gatifloxacin (1-8)	≥8[i]*	≥8[i]*	≥8[i]*	≥8[i]*	≥8[i]*
Gentamicin (2-16 & 500)	16 [a]	16 [a]	16 [a]	16 [a]*	16 [a]
Levofloxacin (0.25-8)	4[h*]	4[h*]	4[h*]	4[h*]	4[h*]
Linezolid (0.5-8)	≥8[d]	≥8[d]	4[c]	≥8[d]*	≥8[e]
Oxacillin+2%NaCl (0.5-8)	8[d]	8[d]*	8[d]*	8[d]*	8[d]
Penicillin (0.06-8)	4[d]	4[d]*	4[d]*	4[d]*	1[e]
Quinupristin/ Dalfopristin (0.12-4)	4[h]	4[h]	4[h]	4[h]	4[h]
Rifampin (0.5-4)	32[gc]	32[gc]	32[c]	32[gc]	4[e]
Streptomycin (1000)	64[a]	64[a]	64[a]	16[a]	64[a]
Tetracycline (2-16)	32[a]	32[a]	8[a]	32[a]	8[a]
Trimethoprim/ Sulfamethoxazole (0.5/9.5-4/76)	32 /512 [d]	>32* /512 [d]	32 /512 [d]	2* /512 [d]	32* /512 [d]
Vancomycin (1-128)	4[g]	4[g]	4[g]	4[g]	≥32[e]

a = EFSA (2012)

b= EFSA (2015)

C = Klayraung *et al*. (2008)

d = Ouoba *et al*. (2008)

e = Florez *et al*. (2016)

f = Humphries *et al*. (2013)

g = ECHCPDG (2003)

h = EFSA (2008)

i= CLSI (2016)

J: British Society for Antimicrobial Chemotherapy (BSAC) Methods for Antimicrobial Susceptibility Testing (2013)

H: European Committee on Antimicrobial Susceptibility Testing 2014

*: breakpoints determined in this study using those of similar genera

Table 6.2b Proposed Breakpoints for the LAB studied

Antimicrobials/ concentration range	LAB/Breakpoints (µg/ml)					
	Leu. lactis	*Weisalla confusa*	*Weisella paramesenteroides*	*St. salivarius*	*St. cristatus*	*Ent. faecium*
Ampicillin (0.12-16)	2[a]	2[e]	2[e]	2[a]*	2[a]*	2[a]
Ceftriaxone (8-64)	4*	4*	4*	4*	4*	4*
Ciprofloxacin (0.5-2)	>32[e]	>32[e]	4*	4*	4*	4[b]
Clindamycin (0.12-1)	1[a]	1[e]	1[e]	2[a]*	2[a]*	4[a]
Daptomycin (0.25-8)	4*	4*	4*	1[j]	1[j]	4[f]
Erythromycin (0.25-4)	1[a]	1[e]	1[e]	2[a]*	2[a]*	4[a]
Gatifloxacin (1-8)	≥8[i]*	≥8[i]*	≥8[i]*	≥8[i]*	≥8[i]*	≥8[i]
Gentamicin (2-16 & 500)	16 [a]	128[d]	128[d]	32 [a]*	32 [a]*	32 [a]
Levofloxacin (0.25-8)	4[h*]	4[h*]	4[h*]	4[h*]	4[h*]	4[h]
Linezolid (0.5-8)	≥8[e]	≥8[de]	≥8[de] *	4[J*]	4[J*]	4[b]
Oxacillin+2%NaCl (0.5-8)	8[d]*	8[d]*	8[d]*	8[d]	8[d]*	8[d]*
Penicillin (0.06-8)	1[e]	1[e]	1[e]	2[J]	2[J]	≥ 16[i]
Quinupristin/ Dalfopristin (0.12-4)	4[h]	4[h]*	4[h]*	4[h]	4[h]*	4[h]
Rifampin (0.5-4)	4[e]	4[e]	4[e]	4*	4*	4[g]
Streptomycin (1000)	64[a]	64[e]	64[e]	64[a*]	64[a*]	128[a]
Tetracycline (2-16)	8[a]	8[e]	8[e]	4[a]*	4[a]*	4[a]
Trimethoprim/ Sulfamethoxazole (0.5/9.5-4/76)	32* /512 [d]	32* /512 [d]	32* /512 [d]	32* /512 [d]	32* /512 [d]	32* /512 [d]
Vancomycin (1-128)	≥32[e]	≥32[e]	≥32[e]	4[a*]	4[a*]	4[a]

a = EFSA (2012)

b= EFSA (2015)

C = Klayraung *et al.,* (2008)

d = Ouoba *et al*., (2008)

e = Florez *et al*., (2016)

f = Humphries *et al*.,(2013)

g = ECHCPDG (2003)

h = EFSA (2008)

i= CLSI (2016)

J: British Society for Antimicrobial Chemotherapy (BSAC) Methods for Antimicrobial Susceptibility Testing (2013)

H: European Committee on Antimicrobial Susceptibility Testing 2014

*: breakpoints determined in this study using those of similar genera

6.2.3 Assessment of antimicrobial resistance genes

Assessment of AMR genes was carried out using polymerase chain reaction (PCR) with specific primers for each AMR gene screened. The AMR genes that were screened are;

1) **Erythromycin**: *erm*(A), *erm*(B) and *erm*(C)
2) **Gentamicin**: *aac*(6″)*aph* (2″), *aac*(3″) V, *ant*(2″)-1
3) **Methicilin**: *mec*A and *mec*C
4) **Penicillin**: *blaz*A
5) **Streptomycin**: *str*A, s*tr*B, *aad*A, *aad*E
6) **Tetracycline**: *tet*(K), *tet*(L),*tet*(M), *tet*(O), *tet*(Q), *tet*(S),*tet*(W)
7) **Vancomycin**: *van*A, *van*B, *van*C

6.2.3.1 DNA extraction

A colony from a 48 h culture was picked and added in 1ml of autoclaved high purity water (Sigma, Gillingham, UK). The suspension was then centrifuged for 1min at 12,000 x g (Eppendorf 5415R, UK). The supernatant was carefully discarded and 100 µl of InstaGene matrix (Bio-Rad, UK) was added to the pellet. This was followed by an incubation of the mixture at 56 ^{o}C for 30 min. Further, the tube was rotated on a vortex (Fisons, UK) for 30 s and placed in a 100 ^{o}C heat block for 8-10 min. After the latter incubation, the tube was rotated again on the vortex for another 30 s and the mixture centrifuged at 12,000 x g (Eppendorf 5415R, UK) for 3 min. The DNA extract in the supernatant was transferred into a sterile eppendorf tube and stored at -20 ^{o}C until required for further analysis.

6.2.3.2 Polymerase chain reaction (PCR) for screening the AMR genes

The presence of AMR genes coding for some AMR specific genes (mentioned above) was screened by PCR using specific primers. For the PCR of erythromycin, gentamicin, tetracycline and vancomycin resistance genes, the 50 µl PCR mixture consisted of 40.3 µl autoclaved high purity water, 5 µl PCR buffer (containing 15 mM of magnesium chloride), 0.5 µl dNTP (1.25 mM), 0.5 µl each of forward and reverse primers (21 pmol/µl), 0.2 µl Taq DNA polymerase (5U) and 3 µl DNA extract. Primers and annealing temperatures for each gene are shown in (Table 6. 3a and 6.3b).

The PCR mixture for streptomycin *str*A and *str*B resistance genes consisted of 38.3 µl autoclaved high purity water, 5 µl PCR buffer (containing 15 mM of with

magnesium chloride), 0.5 µl dNTP (1.25 mM), 2 µl magnesium chloride (25 mM), 0.5 µl each of forward and reverse primers (21pmol/µl), 0.2 µl Taq DNA polymerase (5U) and 3 µl DNA extract.

The PCR mixture for streptomycin *aad*A resistance gene consisted of 39.8 µl autoclaved high purity water, 5 µl PCR buffer(containing 15 mM of with magnesium chloride), 0.5 µl dNTP(1.25 mM), 0.5 µl magnesium chloride (25 mM), 0.5 µl each of forward and reverse primers (21pmol/µl), 0.2 µl Taq DNA polymerase (5U) and 3 µl DNA extract.

The PCR mixture for streptomycin *aad*E and penicillin *blaz*A resistance genes consisted of 37.3 µl autoclaved high purity water, 5 µl PCR buffer (containing 15 mM of with magnesium chloride), 0.5 µl dNTP(1.25 mM), 3 µl magnesium chloride (25 mM), 0.5 µl each of forward and reverse primers (21pmol/µl), 0.2 µl Taq DNA polymerase (5U) and 3 µl DNA extract.

The reaction mixture for methicillin (*Mec*A and *Mec*C) resistance genes was made of 6.5 µl autoclaved high purity water, 12.5 µl 2x green master mix, 2 µl each of forward and reverse primers and 3 µl DNA extract.

The PCR amplifications were carried out in a thermocycler (GeneAmp PCR 2700 system, Applied Biosystems, UK) with the following temperature sequences: initial denaturation of 94 ^{o}C for 3 min, 25 or 35 cycles of 94 ^{o}C for 1 min, annealing temperature of 45 ^{o}C to 67 ^{o}C for individual primers (Table 4) and 72 ^{o}C for 1 min. This was followed by a final extension at 72 ^{o}C for 10 min.

A volume of 10 µl of the amplicons (PCR products) was separated by electrophoresis on 1.5% (w/v) agarose gel. The gel was stained with ethidium bromide (0.5 µl/ml), distained in water and visualized under UV light.

The Taq polymerase, PCR buffer and magnesium chloride were from Applied Biosystems, UK, the high purity water and primers from Sigma, UK and the dNTP mix from Promega, UK.

6.2.3.3 Sequencing of resistances genes

The PCR products of the screened resistance genes that showed visible bands were purified with the Qiagen purification kit (Qiagen, UK) as described by the manufacturer. At the initial stage of using a new purification kit, about 96-100% ethanol was added to buffer PE before usage. An aliquot of 225 µl buffer PB was added to 45 µl of PCR sample in an eppendorf tube and the mixture transferred into a QIAquick spin column placed in a 2 ml collection tube followed by centrifugation at 13,000 x g for 1 min to bind the DNA to the column. The filtered liquid was discarded and750 µl buffer PE added in the column followed by centrifugation (13,000 x g for 1 min) in order to wash the DNA. The filtered liquid was discarded and additional centrifugation step operated to eliminate any residual PE buffer. The column was then transferred in a clean 1.5 ml eppendorf tube and the elute DNA eluted by adding 50 µl buffer EB followed by centrifugation at 13,000 x g for 1 min. The filtered purified DNA obtained was used for the sequencing. The purified products were sequenced (Source bioscience, Cambridge, UK) using the same primers at a volume of 3.2 µl. The

sequences were analysed in the Genbank data base using Basic Alignment Search Tool (BLAST).

Table 6.3a Resistance genes with specific primers and annealing temperatures

Resistance genes	Primers*	Annealing temperature (°C)	Amplicon size (bp)
aac(3″) IV	5′-GTG TGC TGC TGG TCC ACA GC-3′ 5′-AGT TGA CCC AGG GCT GCT GC-3′	63	1550
*aad*A	5′-ATC CTT CGG CGC GAT TTT G-3′ 5′-GCA GCG CAA TGA CAT TCT TG-3′	56	3000
*aad*E	5′-ATG GAA TTA TTC CCA CCT GA-3′ 5′-TCA AAA CCC CTA TTA AAG CC-3′	50	2000
aac(6″)*aph*(2″)	5′-CCA AGA GCA ATA AGG GCA TA-3′ 5′-CAC TAT CAT AAC CAC TAC CG-3′	48	1400
ant(2″)-1	5′-GGG CGC GTC ATG GAG GAG TT-3′ 5′-TAT CGC GAC CTG AAA GCG GC-3′	67	500
*blaz*A	5′-CAGTTCACATGCCAAAGAG -3′ 5′- TACACTCTTGGCGGTTTC -3′	54	2000
erm(A)	5′-AAG CGG TAA AAC CCC TCT GAG-3′ 5′-TCA AAG CCT GTC GGA ATT GG-3′	55	1400
*erm(*B)	5′-CAT TTA ACG ACG AAA CTG GC-3′ 5′-GGA ACA TCT GTG GTA TGG CG-3′	52	1000
erm(C)	5′-CAA ACC CGT ATT CCA CGA TT-3′ 5′-ATC TTT GAA ATC GGC TCA GG-3′	48	1000
*mec*A	5’ – TCCAGATTACAACTTCACCAGG–3’ 5’ – CCACTTCATATCTTGTAACG–3’	59	300
*mec*C	5’ – GAAAAAAAGGCTTAGAACGCCTC–3’ 5’ – GAAGATCTTTTCCGTTTTCAGC–3’	59	200

***** Primers adopted from European Union Reference Laboratory for AMR, National Institute, DTU, Lyngby, Denmark.

Table 6.3b Resistance genes with specific primers and annealing temperatures

Resistance genes	Primers*	Annealing temperature (°C)	Amplicon size (bp)
*str*A	5′-CTT GGT GAT AAC GGC AAT TC-3′ 5′-CCA ATC GCA GAT AGA AGG C-3′	55	1400
*str*B	5′-ATC GTC AAG GGA TTG AAA CC-3′ 5′-GGA TCG TAG AAC ATA TTG GC-3′	56	1400
tet(K)	5′-TTA GGT GAA GGG TTA GGT CC-3′ 5′-GCA AAC TCA TTC CAG AAG CA-3′	55	1550
tet(L)	5′-GTT GCG CGC TAT ATT CCA AA-3′ 5′-TTA AGC AAA CTC ATT CCA GC-3′	54	1550
tet(M)	5′-GTT AAA TAG TGT TCT TGG AG-3′ 5′-CTA AGA TAT GGC TCT AAC AA3′	45	1500
tet(O)	5′-GAT GGC ATA CAG GCA CAG AC-3′ 5′-CAA TAT CAC CAG AGC AGG CT-3′	55	1550
tet(Q)	5′-ATG TTC AAT ATC GGT ATC AAT GA-3′ 5′-GCG GAT ATC ACC TTG CTT C-3′	55	1000
*te*t(S)	5’-TGG AAC GCC AGA GAG GTA TT-3’ 5’-ACA TAG ACA AGC CGT TGA CC-3’	55	1550
tet(W)	5’-GCCATCTTGGTGATCTCC-3’ 5’-TGGTCCCCTAATACATCGTT-3’	55	1550
*van*A	5′-AAC AAC TTA CGC GGC ACT-3′ 5′-AAA GTG CGA AAA ACC TTG C-3′	55	1550
*van*B	5′-GAT ATT CAA AGC TCC GCA GC-3′ 5′-TGA TGG ATG CGG AAG ATA CC-3′	55	1000
*van*X	5′-TGC GAT TTT GCG CTT CAT TG-3′ 5′-ACT TGG GAT AAT TTC ACC GG-3′	55	1400

***** Primers adopted from European Union Reference Laboratory for AMR, National Institute, DTU, Lyngby, Denmark.

6.3 Results

6.3.1 Susceptibility of the LAB studied to antimicrobials

The results of the MIC of various antimicrobials for the different LAB are shown in Table 6.4. All eleven LAB were sensitive to ciprofloxacin, gatifloxacin and quinupristin/dalfopristin as suggested by MIC values that are below the proposed breakpoints. On the other hand, they were all resistant to daptomycin. For the rest of antimicrobial, the susceptibility was variable according to the isolate and the antimicrobial screened. For example, most LAB were sensitive to ampicillin and oxacillin except the isolate of *Enterococcus faecium* which showed resistance to the antimicrobial. Most were also sensitive to penicillin except the isolate of *Weisella paramesentreoides* which was resistant. *Weisella confusa*, *Weisella paramesentreoides* and *Enterococcus faecium* were resistant to rifampin whereas the other isolates were sensitive. For trimethoprim/sulfamethoxazole and streptomycin, it was not possible to determine the susceptibility of the isolates because the range of the concentrations of the antimicrobials included in the MIC did not cover the value of the proposed breakpoints.

Table 6.4a Minimum inhibitory concentration of various antimicrobials for the LAB studied and their associated susceptibility patterns.

Antimicrobials	MIC (µg/ml)				
	Lb. plantarum	*Lb. pentosus*	*Lb. fermentum*	*Lb. coryniformis*	*Leu. mesenteroides* subsp. *mesenteroides*
Ampicillin (0.12-16)	1 S	0.25 S	≤0.12 S	0.25 S	0.5 S
Ceftriaxone (8-64)	≤8 R	8 R	≤8 R	≤8 R	32 R
Ciprofloxacin (0.5-2)	>2 S	>2 S	>2 S	>2 S	>2 S
Clindamycin (0.12-1)	>2 R	>2 R	0.5 S	≤0.12 S	0.25 S
Daptomycin (0.25-8)	>8 R	>8 R	>8 R	>8 R	>8 R
Erythromycin (0.25-4)	4 R	4 R	4 R	2 R	4 R
Gatifloxacin (1-8)	8 S	4 S	>8 S	2 S	4 S
Gentamicin (2-16 & 500)	>16 R	>16 R	>16 R	16 S	8 S
Levofloxacin (0.25-8)	>8 R	>8 R	>8 R	4 S	>8 R
Linezolid (0.5-8)	4 S	2 S	>8 R	4 S	4 S
Oxacillin+2%NaCl (0.5-8)	4 S	4 S	2 S	1 S	2 S
Penicillin (0.06-8)	2 S	1 S	0.25 S	0.25 S	0.25 S
Quinupristin/ Dalfopristin (0.12-4)	2 S	4 S	0.25 S	2 S	1 S
Rifampin (0.5-4)	2 S	2 S	≤0.5 S	≤0.5 S	1 S
Streptomycin (1000)	>1000 R	≤1000NA	≤1000 NA	≤1000 NA	≤1000 NA
Tetracycline (2-16)	>16 R	>16 R	16 R	>16 R	16 R
Trimethoprim/ Sulfamethoxazole (0.5/9.5-4/76)	>4/76NA	>4/76NA	>4/76NA	4/76NA	>4/76 NA
Vancomycin (1-128)	>128 R	>128 R	>128 R	>128 R	>128 R

R = Resistance

S = Susceptibility

NA =Not applicable

Table 6.4b Minimum inhibitory concentration of various antimicrobials for the LAB studied and their associated susceptibility patterns.

Antimicrobials	MIC (µg/ml)					
	Leu. mesenteroides subsp. *lactis*	*Weisella confusa*	*Weisella paramesenteroides*	*St. salivarius* subsp. *thermophilus*	*St. cristatus*	*Ent. faecium*
Ampicillin (0.12-16)	0.25 S	0.25 S	≤0.12 S	≤0.12 S	≤0.12 S	>16 R
Ceftriaxone (8-64)	>64 R	16 R	≤8 S	≤8 S	≤8 S	>64 R
Ciprofloxacin (0.5-2)	>2 S	>2 S	>2 S	>2 S	>2 S	>2 S
Clindamycin (0.12-1)	≤0.12 S	0.5 S	>2 R	>2 R	0.5 S	2 S
Daptomycin (0.25-8)	>8 R	>8 R	>8 R	>8 R	>8 R	>8 R
Erythromycin (0.25-4)	2 R	2 R	4 R	>4 R	2 S	>4 R
Gatifloxacin (1-8)	≤1 S	≤1 S	4 S	2 S	4 S	>8 S
Gentamicin (2-16 & 500)	16 S	>500 R	>16 S	>500 R	>16 S	>500 R
Levofloxacin (0.25-8)	4 S	4 S	>8 R	8 R	>8 R	>8 R
Linezolid (0.5-8)	8 S	4 S	2 S	≤0.5 S	4 S	4 S
Oxacillin+2% NaCl (0.5-8)	1 S	2 S	2 S	≤0.25 S	1 S	>8 R
Penicillin (0.06-8)	≤0.06 S	1 S	2 R	≤0.06 S	1 S	>8 S
Quinupristin/ Dalfopristin (0.12-4)	1 S	0.5 S	4 S	2 S	1 S	0.5S
Rifampin (0.5-4)	1 S	>4 R	>4 R	≤0.5 S	≤0.5 S	>4 R
Streptomycin (1000)	≤1000 NA	≤1000 NA	≤1000 NA	≤1000 NA	>1000 R	>1000 R
Tetracycline (2-16)	4 S	>16 R	8 S	>16 R	>16 R	>16 R
Trimethoprim/ Sulfamethoxazole (0.5/9.5-4/76)	>4/76 NA	>4/76 NA	>4/76 NA	>4/76 NA	>4/76 NA	>4/76 NA
Vancomycin (1-128)	>128 R	>128 R	>128 R	≤1 S	>128 R	4 S

R = Resistance

S = Susceptibility

NA =Not applicable

6.3.2 Determination of AMR genes

The result of the screening of AMR genes is shown in Table 6.5a, 6.5b, and Figure 6.1, 6.2 and 6.3. No positive amplicon was obtained for the genes screened for gentamicin, methicillin, penicillin and streptomycin and vancomycin. For Erythromycin, a positive amplicon for the *erm*(B) was obtained with *Streptococcus salivarius* subsp. *thermophilus* and the identity of the gene was confirmed by sequencing. For tetracycline, the *tet*(K), *tet*(L) and *tet*(M) genes were detected in *Enterococcus faecium* where as, *tet*(M) and *tet*(L) were detected in *Streptococcus salivarius* subsp. *thermophilus* and in *Streptococcus cristatus* respectively. The identity of the pre-cited tetracycline genes was confirmed by sequencing. As seen in Tables 6.5a and 6.5b, positive amplicons were obtained for *erm(*A), *tet*(Q), and *tet*(W) genes but either the bands were faint or their occurrence was not stable over the repeat experiments.

Table 6.5a Antimicrobial resistance gene screening

Isolates	Antimicrobials												
	Erythromycin			Gentamycin			Methicillin		Penicillin	Streptomycin			
	erm(A)	*erm*(B)	*erm*(C)	*ant* (2”)	*aac(*3”)	*aac* (6”) *aph* (2”)	*mec*A	*mec*C	*blaz*A	*str*A	*str*B	*aad*A	*aad*E
1	±	-	-	-	-	-	-	-	-	-	-	-	-
2	-	+	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-	-
4	±	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-	-
6	±	-	-	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-	-	-	-
10	±	-	-	-	-	-	-	-	-	-	-	-	-
11	±	-	-	-	-	-	-	-	-	-	-	-	-

+ = Confirmed gene

+ = Positive PCR

±= initial band faint and repeated PCRs were negative or faint = negative PCR

1 = *Lactobacillus plantarum*

2 = *Streptococcus salivarius* subsp. *thermophilus*

3 = *Leuconostoc mesenteroides* subsp. *mesenteroides*

4 = *Lactobacillus fermentum*

5 = *Weissella paramesenteroides*

6 = *Lactobacillus pentosus*

7 = *Enterococcus faecium*

8 = *Leuconostoc mesenteroides* subsp. *lactis*

9 = *Weissella confusa*

10 = *Streptococcus cristatus*

11 = *Lactobacillus coryniformis*

Table 6.5b Antimicrobial resistance gene screening

Isolates	Antimicrobials									
	Tetracycline							Vancomycin		
	tet(K)	*tet*(L)	*tet*(M)	*tet*(O)	*tet*(Q)	*tet*(S)	*tet*(W)	*van*A	*van*B	*van*X
1	-	-	-	-	±	-	-	-	-	-
2	-	-	+	-	±	-	±	-	-	-
3	-	-	-	-	±	-	±	-	-	-
4	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	±	-	-	-	-	-
6	-	-	-	-	±	-	-	-	-	-
7	+	+	+	-	±	-	-	-	-	-
8	-	-	-	-	±	-	-	-	-	-
9	-	±	-	-	±	-	-	-	-	-
10	-	+	-	-	±	-	±	-	-	-
11	-	±	-	-	±	-	-	-	-	-

+ = Confirmed gene

+ = Positive PCR

±= initial band faint and repeated PCRs were negative or faint = negative PCR

1 = *Lactobacillus plantarum*

2 = *Streptococcus salivarius* subsp. *thermophilus*

3 = *Leuconostoc mesenteroides* subsp. *mesenteroides*

4 = *Lactobacillus fermentum*

5 = *Weissella paramesenteroides*

6 = *Lactobacillus pentosus*

7 = *Enterococcus faecium*

8 = *Leuconostoc mesenteroides* subsp. *lactis*

9 = *Weissella confusa*

10 = *Streptococcus cristatus*

11 = *Lactobacillus coryniformis*

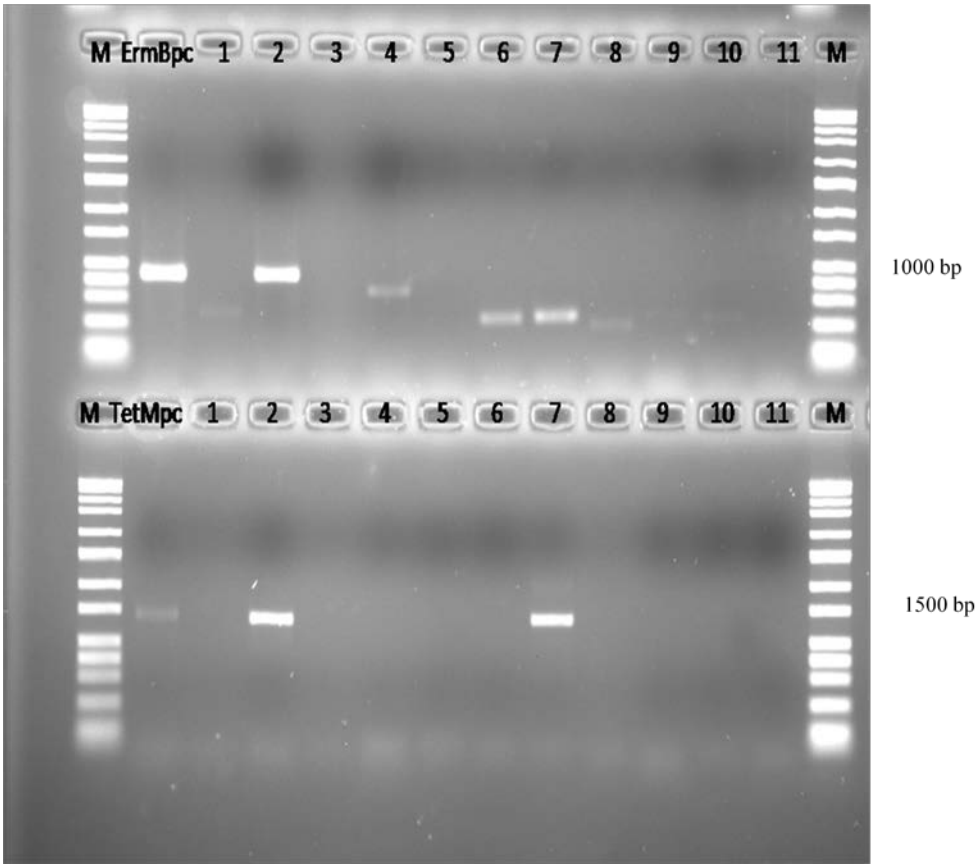

Figure 6.1 Antimicrobial resistance gene gel image of *erm*(B) and *tet*(M)

1 = *Lactobacillus plantarum*

2 = *Streptococcus salivarius* subsp. *thermophilus*

3 = *Leuconostoc mesenteroides* subsp. *mesenteroides*

4 = *Lactobacillus fermentum*

5 = *Weissella paramesenteroides*

6 = *Lactobacillus pentosus*

7 = *Enterococcus faecium*

8 = *Leuconostoc mesenteroides* subsp. *lactis*

9 = *Weissella confusa*

10 = *Streptococcus cristatus*

11 = *Lactobacillus coryniformis*

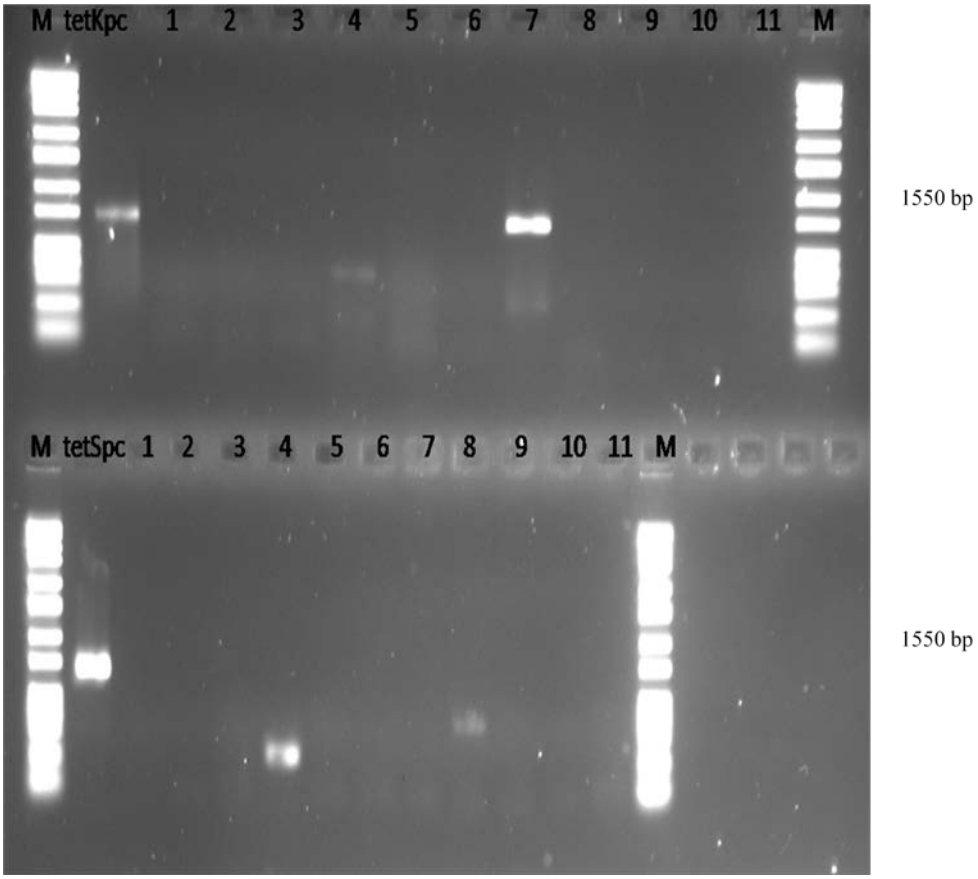

Figure 6.2: Antimicrobial resistance gene gel image of *tet*(K) and *tet*(S)

1 = *Lactobacillus plantarum*

2 = *Streptococcus salivarius* subsp. *thermophilus*

3 = *Leuconostoc mesenteroides* subsp. *mesenteroides*

4 = *Lactobacillus fermentum*

5 = *Weissella paramesenteroides*

6 = *Lactobacillus pentosus*

7 = *Enterococcus faecium*

8 = *Leuconostoc mesenteroides* subsp. *lactis*

9 = *Weissella confusa*

10 = *Streptococcus cristatus*

11 = *Lactobacillus coryniformis*

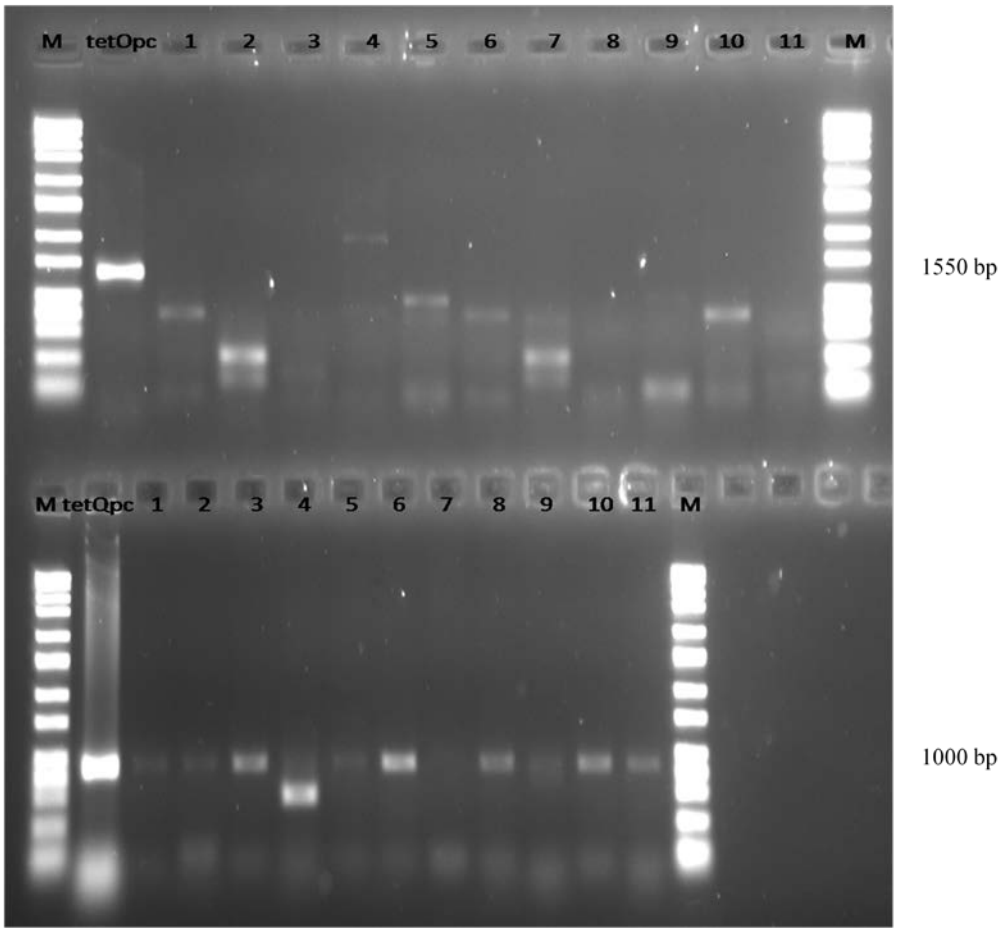

Figure 6.3: Antimicrobial resistance gene gel image of *tet*(O) and *tet*(Q)

1 = *Lactobacillus plantarum*

2 = *Streptococcus salivarius* subsp. *thermophilus*

3 = *Leuconostoc mesenteroides* subsp. *mesenteroides*

4 = *Lactobacillus fermentum*

5 = *Weissella paramesenteroides*

6 = *Lactobacillus pentosus*

7 = *Enterococcus faecium*

8 = *Leuconostoc mesenteroides* subsp. *lactis*

9 = *Weissella confusa*

10 = *Streptococcus cristatus*

11 = *Lactobacillus coryniformis*

6.4 Discussion

For safety of probiotic LAB intended to be used as supplement or in food products, the FAO/WHO (2006) recommends screening for AMR genes. Therefore, the phenotypic and genotypic AMR profile of LAB isolated from human breast milk was investigated in this study. The entire LAB were sensitive to quinolones (ciproflaxicin and gatifloxacin) as well as streptogramins (quinupristin/dalfopristin) indicating inhibition of DNA synthesis. Similarly, the entire LAB were susceptible to beta-lactams (ampicillin, penicillin and oxicillin) with the exception of *Enterococcus faecium* which showed resistance to ampicillin and oxicillin and *Weisella paramesentreoides* which was resistant to penicillin. The beta-lactams are known for their inhibition of cell wall synthesis (Cho *et al*. 2014). The entire LAB were also sensitive to the oxazolidinones (linezolid) with the exception of *Lactobacillus fermentum*. Similar trend was

reported in the study of Florez *et al*. (2016) where *Leuconostoc* and *Weissella* species isolated from fermented dairy milk showed susceptibility towards ciprofloxacin, ampicillin, penicillin and linezolid. Also, similar to the current study, *Lactobacillus* species isolated from human breast milk were susceptible to ampicillin and penicillin (Martin *et al*. 2005, Malek *et al*. 2010, Sharma *et al*. 2014, Kozak *et al*. 2015,Sharma *et al*. 2017) as well as to clindamycin and quinupristin/dalfopristin (Kozak *et al*. 2015). However, Jiang *et al*. (2016) indicated a resistance of lactobacilli isolated from human breast milk to ciproflaxicin. The difference with the current study may be due to various factors such as the origin of the isolates and the screening methods used.

It has been reported that resistance of LAB to vancomycin is an intrinsic trait related to the presence of D-alanyl-D-lactate in their peptidoglycan as opposed to D-alanyl dipeptide which inhibit the binding of vancomycin (Gueimonde *et al*. 2013, Florez *et al*. 2016). In fact, Sharma *et al*. (2017) and Kozak *et al*. (2015) reported vancomycin resistance in *Lactobacillus plantarum* and *Lactobacillus pentosus* from human breast milk. This was also observed for *Leuconostoc* and *Weissella* species from fermented dairy milk (Florez *et al*. 2016). In the current study, the LAB demonstrated high resistance to glycopeptides (daptomycin and vancomycin), but the isolates of *Streptococcus salivarius* subsp. *thermophilus* and *Enterococcus faecium* were susceptible to vancomycin. This study corroborates with the research finding of Jimenez *et al*. (2013) which also reported susceptibility of *Enterococcus faecium* from human breast milk to vancomycin. The results of the current study and that of Jimenez *et al*. (2013) suggest that the

intrinsic resistance to vancomycin claimed for LAB is not applicable to all species. The resistance to other antimicrobials such as ceftriaxone, erythromycin, gentamicin, levofloxacin, tetracycline and clindamycin observed in the current study for some LAB has also been reported in earlier similar studies (Kozak *et al*. 2015, Ouoba *et al*. 2008 and Sharma *et al.* 2017). Tetracyclines are widely used globally in humans and food animals therefore, tetracycline resistance and resistant genes is very common in bacteria (Vries *et al*. 2011 and Jimenez *et al*. 2013).

Multi-drug resistance was observed in the LAB investigated with the highest seen in *Enterococcus faecium* which exhibited resistance to 10 antimicrobials. This trend was observed by Kivanc *et al*. (2016) and Reis *et al*. (2016) who reported multiple drug resistance in *Enterococcus faecium* isolated from human breast milk to ciprofloxacin, ampicillin, gentamicin, penicillin and vancomycin. Also, Munoz *et al.* (2013) observed resistance of *Enterococcus faecium* isolated from aquatic environment to multiple drugs including erythromycin, ciproflaxicin, rifampicin, tetracycline and vancomycin.

The confirmation of the presence of the *tet*(K), *tet*(L), *tet*(M) and *erm*(B) genes in some of the isolates support their phenotypic resistance to tetracycline and erythromycin. The presence of *tet*(M) and *erm*(B) genes observed in the *Streptococcus salivarius* was also reported by Zhang *et al.(*2011) in isolates from the same genus and also from human breast milk. Antibiotic resistance genes including *tet*(M) were found in *Enterococcus* species from the gut of 16 American infants without antibiotic exposure. These infants were fed breast milk or infant

formula from birth to one year (Rolain, 2013). Egervin *et al*. (2009) reported the presence of *tet*(W) gene in *Lactobacillus plantarum* and *Lactobacillus reuteri* isolated from human breast milk. In the current study, faint positive amplicons for *tet*(W) were observed for *Streptococcus cristatus*, *Streptococcus salivarius* subsp. *thermophilus* and *Leuconostoc mesenteroides* subsp. *mesenteroides* but the concentration was not enough to allow a sequencing of the products and the confirmation of the identity of the gene. However, Thummu and Halami (2012) reported the presence of *tet*(W) in *Lactobacillus salivarius* isolated from Indian fermented food. Wang *et al*. (2006) reported *tet*(A) gene in *Streptococcus thermophilus* and *Lactococcus lactis* isolated from cheese. Use of tetracycline in humans and animals has contributed to the persistence of *tet* genes in bacteria (Vries *et al*. 2011 and Jimenez *et al*. 2013). On the other hand, Ouoba *et al*. (2008) reported the presence of *gyr*A (ciprofloxacin) and *aph*(3')II (Kanamycin) resistance genes in *Lactobacillus plantarum, Lactobacillus paraplantarum* and *Lactobacillus casei* isolated from human origin. Sulfonomide resistance gene (*sul*2) was reported in *Enterococcus* and *Streptococcus* species isolated from human breast milk (Zhang *et al*. 2011). In this study, phenotypic resistances observed with some isolates for some antimicrobials were not associated with the presence of the genes screened and coding for the antimicrobials. This may be related to intrinsic resistance to the antimicrobial or the presence of other genes which were not screened. The high phenotypic resistance of LAB to aminoglycosides (e.g.gentamicin), glycopeptides (daptomycin and vancomycin) and tetracycline could be due to selective pressure for resistance to these

antimicrobials. Antimicrobial resistance is a multifaceted problem that could be attributed to various kinds of selection pressures and mode of transmission (Ouoba *et al*. 2008).

Many studies have suggested that some beneficial bacteria such as bifidobacteria and LAB originate from maternal gut (Martin *et al*. 2003, Fernandez *et al.* 2004, Gueimonde*et al.* 2007, Perez *et al*. 2007, Jimenez *et al.* 2008, Arroyo *et al*. 2010, Fernandenz *et al.* 2013, Jeurink *et al*. 2013). Supporting this theory, the presence of AMR and AMR genes in the LAB screened could be related to translocation of LAB with these features from maternal gut to mammary glands. However, if this theory is to be opposed, then presence of the *tet*(K), *tet* (L), *tet*(M) and *erm*(B) genes observed in this study could be attributed to contaminated maternal nipples that could harbour bacteria with potential AMR genes. Maternal gastrointestinal tract and skin microflora are possible reservoirs of antimicrobial resistant bacteria which could be transmitted to newborns and infants (Kozak *et al*. 2015). None of the maternal nipples was cleaned with sterilised swabs before collection of the breast milk samples. Cleaning of breast before breast feeding amongst lactating mothers is rarely practice in both developed and developing worlds. In order to have an insight into what babies are ingesting, this study decided to collect samples of breast milk the way babies suckle.

High resistance of LAB to antimicrobials as demonstrated by this study could be attributed to antibiotic misuse and high level of exposure to antimicrobials by women of childbearing age as well as lactating mothers. This is further coupled with the food chain because once a bacterium with resistance to antimicrobials is

ingested, it could be passed to the gut. This could be transferred to infants via breast milk. Lactic acid bacteria that originate from the gut have diverse AMR that could be transferred to other bacteria present in the gut (Rolain, 2013). Frequent isolation of antimicrobial resistant microorganisms in ready to eat food products indicates possibility of disseminating antimicrobial resistant bacteria from food to humans (Zhang *et al*. 2011). Antibiotic residues are frequently found in edible animal flesh and dairy products in Nigeria (Kabir *et al*. 2004, Adetunji, 2011). Also, erythromycin, gentamicin, penicillin, streptomycin and tetracycline and other antimicrobials are inappropriately used for prophylaxis and therapeutics in poultry laying eggs (Adebowale *et al*. 2016 and Mund *et al*. 2017). In Nigeria as well as other developing countries, self medication with various antibiotics is common. Gentamicin, streptomycin, tetracycline and vancomycin and other antimicrobials are easily purchased over the counter without prescription. Lack of proper legislation and regulation contribute significantly to irrational antibiotic prescription and self medication. Thus, inevitably increase resistance of bacteria to antimicrobials (Olayemi *et al*. 2010, Sapkota *et al*. 2010 and Akinyandemu and Akinyandemu, 2014).

6.5 Conclusion

The study was aimed at assessing the phenotypic and genotypic AMR profiles of LAB from breast milk collected from some Nigerian nursing mothers. The results indicate resistance of some of the LAB to diverse antimicrobials, especially to tetracycline, vancomycin, streptomycin and gentamycin. Some LAB also demonstrated susceptibility profile to some of the antimicrobials especially

ciprofloxacin, gatifloxacin and quinupristin/dalfopristin. Positive amplicons were not observed for gentamicin, methicillin, penicillin and streptomycin and vancomycin genes. AMR genes *tet*(K), *tet*(L) and *tet*(M)), *erm*(B) coding for tetracyline and erythromycin were confirmed in *Enterococcus faecium*, *Streptococcus salivarius* subsp. *thermophilus* and *Streptococcus cristatus*. The study has therefore revealed the antimicrobial resistance profile and antimicrobial resistance genes of some LAB isolated from samples of Nigerian human breast milk.

CHAPTER SEVEN: DEMOGRAPHIC STUDY OF HUMAN BREAST MILK SAMPLES WITH RELATION TO LACTIC ACID BACTERIAL POPULATION AND DIVERSITY

7.1 Introduction

Human breast milk is a complex biological fluid that adequately nourishes babies and infants. Human breast milk contain bioactive compounds such as human alpha-lactalbumin made lethal to tumour cells (HAMLET), immune cells, immunoglobulins, lactoferrin, lysozymes, and oligosaccharides (Admyre *et al*. 2007, Mossberg *et al*. 2010, Hakansson *et al*. 2011, Marks *et al*. 2012 and Marks *et al*. 2013). Apart from nutritional components of breast milk, it also has commensal bacteria that are important for development of infant's gut. Some beneficial bacteria such as lactic acid bacteria play a significant role in establishment of intestinal microflora of breast-fed babies. Probiotic LAB improve health of babies by reducing risks of diseases associated with allergy and diarrhea (Gueimonde *et al*. 2007, Martin *et al*. 2009 and Arboleye *et al*. 2011). However, gestational age, lactation stage, maternal diet, nutritional status, geograhical location, mode of delivery and use of antibiotics could affect population and diversity of human breast milk microbiota (Mills, *et al*. 2011, Khodayar-Pardo *et al*. 2014 and Gomez-Gallego *et al*. 2016).

Human milk oligosaccharides (HMOs) stimulate growth of beneficial bacteria, HMOs are therefore prebiotic that promote growth and proliferation of probiotic bacteria (Mills, *et al*. 2011). Moreover, maternal HMOs vary in quality and quantity in relation to lactation period, maternal Lewis blood group and secretor status. But diet, ethnicity, lifestyle and many factors could contribute to structural variations of HMOs (Thum *et al*. 2012). Futhermore, fructo- oligosaccharides (found in onions, asparagus, tomatoes and other vegetables) and galacto-

oligosaccharides are both soluble alimentary fibres. These types of fibres get into gastrointestinal tract where they are fermented by bacteria (Mills, *et al*. 2011). Consumption of prebiotics by lactating mothers could therefore increase population and diversity of beneficial bacteria (Mills, *et al*. 2011).

The aim of the study described in this chapter was to examine breast milk donors' demographic characteristics that could be related with composition of bacterial population and diversity in their breast milk samples. The specific objective of this study was to determine demographic factors (stage of lactation, gender of lactating babies, number of children and diet) that could relate to population and diversity of lactic acid bacteria in human breast milk.

7.2 Materials and methods

7.2.1 Data collection

The instrument for data collection used in this study was questionnaire (Appendix 3). The questionnaires were administered to donors at the time of breast milk collection (2.2.1, donor recruitment). The survey includes relevant questions regarding demographics of donors and their babies as well as donors' diets. The questions were simple and required less than 20 minutes to answer.

7.2.2 Data analysis

Descriptive statistic was used to get the percentage of all the variables of demography of each sample as they relate to bacterial population and diversity. The methods of Arora *et al*. (2000), Odindo *et al*. (2014) and *Patel et al*. (2015) were used for this study.

7.3 Results

7.3.1 Bacterial population and diversity of human breast milk samples

About 18.75% of the samples had a diversity of three kinds of bacteria, 56.25% had two types of bacteria while 25% had one bacterium. The population of the bacteria range from 2.30±0.15 to 4.14±0.13. Samples F and I had less than 97% identity (Table 7.1).

Table 7.1 Bacterial population and diversity of human breast milk samples

Sample	Bacterial counts (cfu/ml) MRS	Bacterial counts (cfu/ml) MRScys	Identified bacteria	% identity
A	3.34±0.22	3.23±0.14	*Enterococcus faecium*	99.39
B	<1x10	<1x10	-	-
C	3.17±0.10	3.23±0.14	*Enterococcus faecium*	99.79
			Leuconostoc mesenteroides subsp. *mesenteroides*	100
D	3.36±0.13	3.27±0.20	*Weissella paramesenteroides*	99.80
			Weissella confusa	99.79
E	3.17±0.10	3.11±0.17	*Lactobacillus fermentum*	99.17
			Staphylococcus epidermidis	100
F	4.04±0.20	2.96±0.35	*Staphylococcus epidermidis*	95.45
G	2.86±0.22	2.30±0.15	*Enterococcus faecium*	99.37
			Staphylococcus epidermidis	99.56
			Staphylococcus hominis	100
H	2.30±0.23	2.49±0.15	*Staphylococcus epidermidis*	100
			Staphylococcus hominis	99.57
I	3.90±0.30	2.92±0.20	*Pantoea dispersa*	89.84
J	2.65±0.29	4.14±0.13	*Weissella paramesenteroides*	99.60
			Lactobacillus pentosus	100

MRS = de Man Rogosa Sharpe agar

MRS-cys = de Man Rogosa Sharpe agar + L-cysteine

cfu/ml = colony forming unit per milliliter

Data are mean $\log_{10}$ ± standard deviation of duplicate experiments

Table 7.1 continued

Sample	Bacterial counts (cfu/ml) MRS	Bacterial counts (cfu/ml) MRScys	Identified bacteria	% identity
K	2.77±0.33	2.96±0.29	*Staphylococcus epidermidis*	97.33
			Staphylococcus epidermidis	99.79
L	2.65±0.10	2.84±0.14	*Lactobacillus pentosus*	97.88
			Streptococcus salivarus subsp. *thermophilus*	100
			Weissella confusa	99.54
M	2.70±0.17	2.94±0.17	*Weissella confusa*	99.77
N	<1x10	<1x10	-	-
O	2.47±0.22	<1x10	*Lactobacillus fermentum*	99.60
P	3.25±0.11	3.34±0.16	*Weissella paramesenteroides*	99.80
			Lactobacillus plantarum	100
Q	3.17±0.10	3.20±0.12	*Staphylococcus hominis*	98.38
			Leuconostoc mesenteroides subsp. *lactis*	99.52
			Streptococcus pneumoniae	82.42
			Streptococcus cristatus	99
R	2.98±0.48	2.14±0.24	*Staphylococcus epidermidis*	100
			Leuconostoc mesenteroides subsp. *lactis*	100
S	3.07±0.10	3.27±0.11	*Staphylococcus epidermidis*	100
			Streptococcus salivarus subsp. *thermophilus*	100
T	3.04±0.21	2.93±0.29	*Lactobacillus pentosus*	100
			Lactobacillus coryniformis	99.79

MRS = de Man Rogosa Sharpe agar

MRS-cys = de Man Rogosa Sharpe agar + L-cysteine

cfu/ml = colony forming unit per milliliter

Data are mean $\log_{10}$ ± standard deviation of duplicate experiments

7.3.2 Relationship between lactation stage (age of babies) and bacterial counts

Sixty percent of the babies' lactation age was between 0-6 months and the population of bacteria isolated from the breast milk was between 2.14± 0.24 to 4.04±0.20 cfu/ml but two samples were >100 cfu/ml. Twenty-five percent of the babies age while lactating was between7-13 months, the colony counts were in the range of 2.30±0.15 to 3.25±0.11 cfu/ml. Babies lactating between 14-20 months were ten percent with bacterial counts between 2.65±0.10 to 4.14±0.13. Those over 20 months were only five percent with bacterial counts of between 3.37±0.20 to 3.36±0.13 (Table 7.2a and 7.2b).

Table 7.2a Relationship between lactation stage (age of babies) and bacterial counts

Lactation stage (Months)	0-6			7-13		
Bacterial counts (cfu/ml)	Sample	MRS	MRScys	Sample	MRS	MRScys
	A	3.34±0.22	3.23±0.14	G	2.86±0.22	2.30±0.15
	B	<1x10	<1x10	H	2.30±0.23	2.49±0.15
	C	3.17±0.10	3.23±0.14	O	2.47±0.22	<1x10
	E	3.17±0.10	3.11±0.17			
	F	4.04±0.20	2.96±0.35	P	3.25±0.11	3.34±0.16
	I	3.90±0.30	2.92±0.20	Q	3.17±0.10	3.20±0.12
	K	2.77±0.33	2.96±0.29			
	M	2.70±0.17	2.94±0.17			
	N	<1x10	<1x10			
	R	2.98±0.48	2.14±0.24			
	S	3.07±0.10	3.27±0.11			
	T	3.04±0.21	2.93±0.29			

MRS = de Man Rogosa Sharpe agar

MRS-cys = de Man Rogosa Sharpe agar + L-cysteine

cfu/ml = colony forming unit per milliliter

Data are mean $\log_{10}$ ± standard deviation of duplicate experiments

Table 7.2b Relationship between Lactation stage (age of babies) and bacterial counts

Lactation stage (Months)	14-20			Over 20		
Bacterial counts (cfu/ml)	Sample	MRS	MRScys	Sample	MRS	MRScys
	J	2.65±0.29	4.14±0.13	D	3.36±0.13	3.27±0.20
	L	2.65±0.10	2.84±0.14			

MRS = de Man Rogosa Sharpe agar

MRS-cys = de Man Rogosa Sharpe agar + L-cysteine

cfu/ml = colony forming unit per milliliter

Data are mean $\log_{10}$ ± standard deviation of duplicate experiments

7.3.3 Relationship between babies' sex and bacterial counts

Male babies represent sixty percent of the babies, the bacterial counts of their mothers' breast milk was between 2.14±0.24 to 4.04±0.20 cfu/ml. Forty percent of the babies were females and the colony counts of their mothers' breast milk was between 2.30±0.23 to 3.90±0.30 cfu/ml but two samples were <100 (Table 7.3).

Table 7.3 Relationship between babies' sex and bacterial counts

Sex of babies	Males			Females		
Bacterial counts (cfu/ml)	Sample	MRS	MRScys	Sample	MRS	MRScys
	A	3.34±0.22	3.23±0.14	B	<1x10	<1x10
	C	3.17±0.10	3.23±0.14	D	3.36±0.13	3.27±0.20
	F	4.04±0.20	2.96±0.35	E	3.17±0.10	3.11±0.17
	G	2.86±0.22	2.30±0.15	H	2.30±0.23	2.49±0.15
	J	2.65±0.29	4.14±0.13	I	3.90±0.30	2.92±0.20
	L	2.65±0.10	2.84±0.14	K	2.77±0.33	2.96±0.29
	M	2.70±0.17	2.94±0.17	N	<1x10	<1x10
	O	2.47±0.22	<1x10	Q	3.17±0.10	3.20±0.12
	P	3.25±0.11	3.34±0.16			
	R	2.98±0.48	2.14±0.24			
	S	3.07±0.10	3.27±0.11			
	T	3.04±0.21	2.93±0.29			

MRS = de Man Rogosa Sharpe agar

MRS-cys = de Man Rogosa Sharpe agar + L-cysteine

cfu/ml = colony forming unit per milliliter

Data are mean $\log_{10}$ ± standard deviation of duplicate experiments

7.3.4 Relationship between number of donors' children and bacterial counts

Forty percent of the donors have only one child and bacterial counts of their breast milk samples was between 2.14±0.24 to 4.04±0.20cfu/ml. Donors with four to five children represented twenty percent of the population and their breast milk bacterial counts was between 2.30±0.15to 3.17±0.10 cfu/ml, but two samples were >100 cfu/ml. Donors with children between 6 to7 and 8 to 9 were fifteen percent and had colony counts of 2.47±0.22 to 3.34±0.16 cfu/ml and 2.65±0.29 to 4.14±0.13 cfu/ml respectively. Donors with children between 2 to 3 and 10-11 constitute five percent of the sampled population with bacterial counts ranging from 3.27±0.20 to 3.36±0.13 cfu/ml and 3.17±0.10 to 3.20±0.12 cfu/ml respectively (Table 7.4a, 7.4b and 7.4c).

Table 7.4a Relationship between number of donors' children and bacterial counts

Number of children		1			2-3	
Bacterial counts (cfu/ml)	Sample	MRS	MRScys	Sample	MRS	MRScys
	C	3.17±0.10	3.23±0.14	D	3.36±0.13	3.27±0.20
	F	4.04±0.20	2.96±0.35			
	H	2.30±0.23	2.49±0.15			
	K	2.77±0.33	2.96±0.29			
	L	2.65±0.10	2.84±0.14			
	R	2.98 ±0.48	2.14±0.24			
	S	3.07±0.10	3.27 ±0.11			
	T	3.04 ±0.21	2.93 ±0.29			

MRS = de Man Rogosa Sharpe agar

MRS-cys = de Man Rogosa Sharpe agar + L-cysteine

cfu/ml = colony forming unit per milliliter

Data are mean $\log_{10}$ ± standard deviation of duplicate experiments

Table 7.4b Relationship between number of donors' children and bacterial counts

Number of children	4-5			6-7		
Bacterial counts (cfu/ml)	Sample	MRS	MRScys	Sample	MRS	MRScys
	B	>100	>100	M	2.70±0.17	2.94±0.17
	E	3.17±0.10	3.11±0.17	O	2.47±0.22	<1x10
	G	2.86±0.22	2.30±0.15	P	3.25±0.11	3.34±0.16
	N	<1x10	<1x10			

Table 7.4c Relationship between number of donors' children and bacterial counts

Number of children	8-9			10-11		
Bacterial counts (cfu/ml)	Sample	MRS	MRScys	Sample	MRS	MRScys
	A	3.34±0.22	3.23±0.14	Q	3.17±0.10	3.20±0.12
	I	3.90±0.30	2.92±0.20			
	J	2.65±0.29	4.14±0.13			

MRS = de Man Rogosa Sharpe agar

MRS-cys = de Man Rogosa Sharpe agar + L-cysteine

cfu/ml = colony forming unit per milliliter

Data are mean $\log_{10}$ ± standard deviation of duplicate experiments

7.3.5 Relationship between donors' diet and bacterial counts

Although donors eat almost similar staple foods, there was still a variation in the bacterial counts of their breast milk samples. The highest counts of 4.04±0.20and 4.14±0.13 cfu/ml were from donors that consume tuwon shinkafa (mashed rice) with vegetable soup and danwake (boiled dumpling) with fried onion and pepper respectively (Table 7.5).

Table 7.5 Relationship between donors' diet and bacterial counts

Samples	Typical daily foods of donors	Bacterial counts (cfu/ml)	
		MRS	MRScys
A	Pounded yam (mashed yam) with egusi (melon seed) soup.	3.34±0.22	3.23±0.14
B	Tuwon shinkafa (mashed rice) with vegetable soup.	<1x10	<1x10
C	Beans and rice with stew	3.17±0.10	3.23±0.14
D	Jollof rice.	3.36±0.13	3.27±0.20
E	Beans, semovita (semolina) fufu with draw soup (ewedu, okra or ogbono).	3.17±0.10	3.11±0.17
F	Tuwon shinkafa (mashed rice) with vegetable soup.	4.04±0.20	2.96±0.35
G	Tuwo (mashed rice, corn or semovita) with kuka (baobab leaf powder) soup.	2.86±0.22	2.30±0.15
H	Tuwo (mashed rice, corn or semovita) with vegetable soup.	2.30±0.23	2.49±0.15
I	Amala (cassava or yam) fufu with vegetable, tea and bread	3.90±0.30	2.92±0.20
J	Danwake (boiled dumpling made from a mixture of bean flour, cassava flour, wheat flour baobab powder and potash) with fried onion and pepper.	2.65±0.29	4.14±0.13
K	Jollof rice.	2.77±0.33	2.96±0.29
L	Rice and stew	2.65±0.10	2.84±0.14
M	Rice and stew	2.70±0.17	2.94±0.17
N	Maize fufu with vegetable soup.	<1x10	<1x10
O	Maize fufu with vegetable soup or kuka (baobab leaf powder) soup.	2.47±0.22	<1x10
P	Tuwon shinkafa (mashed rice) with vegetable soup.	3.25±0.11	3.34±0.16
Q	Rice and stew	3.17±0.10	3.20±0.12
R	Pasta and vegetables	2.98±0.48	2.14±0.24
S	Rice and stew	3.07±0.10	3.27±0.11
T	Rice and beans stew	3.04±0.21	2.93±0.29

MRS = de Man Rogosa Sharpe agar

MRS-cys = de Man Rogosa Sharpe agar + L-cysteine

cfu/ml = colony forming unit per milliliter

Data are mean $\log_{10}$ ± standard deviation of duplicate experiments

7.3.6 Demography of donors and bacterial diversity of human breast milk samples

About 11.11% of donors with age range between 15-25 years had a diversity of three kinds of bacteria, 66.66% had two types of bacteria and 22.22% had one bacterium. About 33.33% of donors with age range between 26-36 years had a diversity of two types of bacteria and 66.66% had one bacterium. Fifty percent of over 36 years donors had diversity of three kinds of bacteria and 50% and had a diversity of two types of bacteria (Table 7.6).

About 62.5% of 0-6 month lactation stage had a diversity of two kinds of bacteria and 37.5% had one bacterium. Forty percent of 7-13 month lactation stage had a diversity of three kinds of bacteria, also 40% had two types of bacteria and 20% had one bacterium. Fifty percent of 14-20 month lactation stage had diversity of three kinds of bacteria and 50% two types of bacteria. Only one sample had a lactation stage of over 20 month and had a hundred percent two types of bacteria.

About 18.18% of donors with male babies had diversity of three kinds of bacteria, 54.54% had two types of bacteria and 27.27% had one bacterium. Forty percent of donors with female babies had diversity of three kinds of bacteria also 40% had two types of bacteria and 20% had one bacterium.

About 14.28% of the mothers with only one child had diversity of three kinds of bacteria, 71.43% had two types of bacteria and 14.28% had one bacterium. Fifty percent of the mothers with 4-5 children had diversity of three kinds of bacteria and 50% had two types. About 33.33% of the mothers with 6-7 children had diversity of two kinds of bacteria and 66.66% had one bacterium. While 50% of

donors with 8-9 children had diversity of two kinds of bacteria and 50% had one bacterium. Donors with children between 2 to 3 and 10-11 had 100% diversity of two kinds of bacteria and three types of bacteria respectively.

Sample L had the highest lactic acid bacterial diversity with *Lactobacillus pentosus*, *Streptococcus salivarus* subsp. *thermophilus* and *Weissella confusa*. The donor was at 14 to 20 months lactation stage, has one child, gender was male and frequently consumed food was rice and stew. Samples C, D, J, P, Q and T each had two types of lactic acid bacteria (Table 7.6).

Table 7.6 Demography of donors and bacterial diversity of human breast milk samples

Sample	Demography	Bacteria
A	0-6 month lactation stage, male, 8-9 children, diet: Pounded yam (mashed yam) with egusi (melon seed) soup.	*Enterococcus faecium*
C	0-6 month lactation stage, male, one child, diet: Beans and rice with stew.	*Enterococcus faecium* *Leuconostoc mesenteroides* subsp. *mesenteroides*
D	Over 20 month, female, 2-3 children, diet: Jollof rice.	*Weissella paramesenteroides* *Weissella confusa*
E	0-6 month lactation stage, female, 4-5 children, diet: Beans, semovita (semolina) fufu with draw soup (ewedu, okra or ogbono).	*Lactobacillus fermentum* *Staphylococcus epidermidis*
G	7-13 month lactation stage, male, 4-5 children, diet: Tuwo (mashed rice, corn or semovita) with kuka (baobab leaf powder) soup.	*Enterococcus faecium* *Staphylococcus epidermidis* *Staphylococcus hominis*
H	7-13 month lactation stage, female, one child, diet: Tuwo (mashed rice, corn or semovita) with vegetable soup and noodles.	*Staphylococcus epidermidis* *Staphylococcus hominis*
J	14-20 month lactation stage, male, 8-9 children, diet: Danwake (boiled dumpling made from a mixture of bean flour, cassava flour, wheat flour baobab powder and potash) with fried onion and pepper.	*Weissella paramesenteroides* *Lactobacillus pentosus*
K	0-6 month lactation stage, male, one child, diet: Jollof rice.	*Staphylococcus epidermidis* *Staphylococcus epidermidis*

Table 7.6 continued

Sample	Demography	Bacteria
L	14-20 month lactation stage, male, one child, diet: Rice and stew	*Lactobacillus pentosus* *Streptococcus salivarus* subsp. *thermophilus* *Weissella confusa*
M	0-6 month lactation stage, male, 6-7 children, diet: Rice and stew	*Weissella confusa*
O	0-6 month lactation stage, male, 6-7 children, diet: Maize fufu with vegetable soup or kuka (baobab leaf powder) soup.	*Lactobacillus fermentum*
P	7-13 month lactation stage, male, 6-7 children, diet: Tuwon shinkafa (mashed rice) with vegetable soup.	*Weissella paramesenteroides* *Lactobacillus plantarum*
Q	7-13 month lactation stage, female, 10-11 children, diet: Rice and stew.	*Staphylococcus hominis* *Leuconostocmesenteroides* subsp. *lactis* *Streptococcus cristatus*
R	0-6 month lactation stage, male, one child, diet: Pasta and vegetables.	*Staphylococcus epidermidis* *Leuconostoc mesenteroides* subsp. *lactis*
S	0-6 month lactation stage, male, one child, diet: Rice and stew	*Staphylococcus epidermidis* *Streptococcus salivarus* subsp. *thermophilus*
T	0-6 month lactation stage, male, one child, diet: Rice and beans stew.	*Lactobacillus pentosus* *Lactobacillus coryniformis*

7.4 Discussion

Demographic factors (stage of lactation, gender of lactating babies, number of children and diet) that could relate to population and diversity of lactic acid bacteria in human breast milk were examined. The population of 10^2 to 10^4 cfu/ml bacterial cells in this study is similar to the counts observed by Martin *et al*. (2003) and Albesharat *et al*. (2011). Other studies have reported LAB population of about 10^3 to 10^4 cfu/ml from the breast milk of healthy mothers (Jeurink *et al*. 2013). A diversity of eleven LAB and two *Staphylococcus* species were present in HBM samples analysed in this study. The highest diversity of three kinds of LAB was observed in only one sample, other samples demonstrated presence of two or one LAB or a mixture of LAB and *Staphylococcus* species. Other samples had only *Staphylococcus* species. Collado *et al*. (2009) reported diversity of *Staphylococcus, Bifidobacterium, Lactobacillus, Enterococcus* and *Streptococcus* species in all 50 samples of Spanish HBM using quantitative real-time PCR technique (qRTI-PCR). This diversity is similar with our findings with the exception of *Bifidobacterium* species which was not present in any of the samples analysed in this study. Similarly, Soto *et al*. (2014) analysed 66 samples of HBM of German and Austrian mothers using PCR. They reported diversity of *Staphylococcus, Streptococcus, Bifidobacterium* and *Lactobacillus* species with diversity of two kinds of species in seven samples. Additionally, Martin *et* a*l*. (2007) reported diversity of LAB from five samples of Spanish HBM using PCR and denaturing gradient gel electrophoresis (DGGE). A diversity of *Lactobacillus fermentum, Weissella confusa, Leuconostoc citreum, Lactobacillus rhamnosus,*

Lactobacillus plantarum and *Leuconostoc fallax* were detected. They observed a diversity of three kinds of LAB in three samples. However, Urbaniak *et al*. (2016) reported diversity of *Staphylococcus, Enterobacteriaceae* and Pseudomonas species in all 39 samples of Canadian HBM. *Streptococcus* and *Lactobacillus* species were present in some samples.

Lactation period of maternal HBM samples did not indicate much difference in the population and diversity of LAB in this study. Bacterial population count of 10^2 and 10^3 cfu/ml were all observed at all stages of lactation. Moreover, cell count of 10^4 was observed in maternal breast samples at 0-6 months and over 20 months lactation period. Similarly, lactation stage did not affect bacterial composition, as *Enterococcus faecium* was present in maternal breast samples at lactation stage of 0-6 months and 7-13 months. Other LAB and *Staphylococcus* species were also present at almost all stages of lactation. This study is in agreement with the study of Sakwanska *et al*. (2016) which indicated no difference in *Bifidobacterium, Lactobacillus, Streptococcus* and *Staphylococcus* species at 4 days to 2 month lactation stage from HBM of Chinese lactating mothers. The findings of Khodayar-Pardo *et al*. (2014) is also in agreement with this study, their result indicate no difference in *Bifidobacterium, Lactobacillus, Enterococcus* and *Staphylococcus* composition of colostrum and mature milk of Spanish lactating mothers. In contrast to this study herein reported, Cabrera-Rubio *et al*. (2012) reported changes in bacterial composition at 0, 1 and 6 months of lactation. They demonstrated high presence of *Lactococcus, Leuconostoc, Staphylococcus, Streptococcus* and *Weissella* species in colostrum sample than in

mature milk samples of Finnish lactating mothers. Similarly, Collado *et al*., (2012) reported high presence of *Enterococcus* species in colostrum than in samples at 1 to 6 month of lactation from Finnish lactating mothers.

In this study, no much difference was also observed in population and diversity of LAB with relation to babies' sex and number of donors' children. Moreover, Urbaniak *et al*. (2016) indicated no difference in population and diversity of *Streptococcus, Lactobacillus, Staphylococcus* and *Pseudomonas* species with relation to infant gender.

Maternal diet could have effect on diversity of LAB as observed in this study. Donors whose diet include a combination of rice, beans, stew and vegetable soup had a diversity of two LAB with one sample demonstrating a diversity of three LAB. Moreover, vegetables especially onions and tomatoes are fructo-oligosaccharides which are easily utilised by some LAB present in GIT (Mills, *et al*. 2011). Furthermore, diet rich in carbohydrate and protein facilitate proliferation of anaerobic bacteria in GIT (Walker and Iyenger, 2015). However, frequent ingestion of antibiotics and consumption of less balanced diet by lactating mothers could contribute to less population and diversity of LAB in human breast milk (Gomez-Gallego *et al*. 2016). Studies on relationship between maternal diet, lactation stage, babies sex and number of maternal children and population and diversity of LAB are very few. But dietary habits could potentially modify intestinal microbiota which consequently affect type of bacteria transferred from maternal gut to mammary gland (Gomez-Gallego *et al*. 2016).

More extensive research on factors that could influence lactic acid bacterial population and diversity in HBM is still needed to provide additional knowledge in this area. This demographic study is weak because lactic acid bacterial diversity was not appropriately measured and dietary information was not sufficient.

7.5 Conclusion

Donors' demographic factors that could influence composition of bacterial population and diversity in their breast milk samples were investigated. A population of 10^2 to 10^4 cfu/ml bacterial cells and diversity of eleven LAB and two *Staphylococcus* species were present in HBM samples examined in this study. No difference was observed in the population and diversity of LAB with relation to lactation stage, babies' sex and number of donors' children. Maternal diet is an important factor that could influence population and diversity of LAB in human breast milk.

CHAPTER EIGHT: GENERAL DISCUSSION AND RECOMMENDATION

8.1 Introduction

This research enumerated, isolated and identified lactic acid bacteria present in some samples of Nigerian human breast milk. It further studied the probiotic characteristics of some of the LAB and resistance of the LAB to some antimicrobials. Finally the research studied the relationship between breast milk donors' demographics with population and diversity of LAB. The overall findings of the research as they relate to their specific aims and objectives are summerised and discussed. Furthermore, strengths and limitations of the research were explained. Recommendations for future studies were also enumerated.

8.2 Scope of the research

To achieve the aims and objectives of this research, the research was conducted in four stages;

- Enumeration, isolation and identification of isolates
- Probiotic characterisation of identified LAB
- Antibiotic resistance study
- Demographic study of human breast milk samples with relation to lactic acid bacterial population and diversity

8.3 Enumeration, isolation and identification of isolates

In order to achieve the aims of the first stage of the research, 20 breast feeding mothers who signed the consent forms were recruited for sample collection in Nigeria (2.2.2). Enumeration of LAB was carried out on MRS and MRS

supplemented with 0.5g/L L-cysteine hydrochloride (2.1.3). Both medium were suitable for growth of LAB. Similarly, Kavitha and Devasena (2013) and Nayra *et al*. (2013) also used the two media for isolation of LAB from human breast milk. However, Diaz-Ropero *et al*. (2006) and Olivares *et al*. (2006) isolated LAB from human breast milk using only MRS agar.

The number of colonies observed on the two media was similar, 2.30±0.23 to 4.04±0.20 cfu/ml on MRS agar and 2.14±0.24 to 4.14±0.13 cfu/ml on MRS L-cysteine hydrochloride agar. Other authors also reported presence of 10^2 to 10^4 cfu/ml cultivable bacteria including LAB in human breast milk (Perez *et al.* 2007 and Martin *et al*. 2009). On the other hand, presence of 10^3 to 10^5 cfu/ml lactic acid bacteria in human breast milk was reported by Martin *et al*. (2003) and Jeurink *et al*. (2013). Recovered isolates from this study were stored in cryovials, they were subsequently purified and stored for identification.

The first step in the identification of the isolates was the phenotypic assessment which involved Gram staining, catalase and oxidase tests. A total of 108 recovered isolates were phenotypically identified. The second stage in the identification consisted of rep-PCR and 16S rRNA gene sequencing. The initial genotypic identification (rep-PCR) facilitated grouping of isolates into 19 groups. The 16S rRNA gene sequencing identified some LAB and staphylococci. These are *Lactobacillus plantarum, Lactobacillus pentosus, Lactobacillus coryniformis, Lactobacillus fermentum, Leuconostoc mesenteroides* subsp. *mesenteroides, Weissella confusa, Weissella paramesenteroides, Streptococcus salivarius* subsp. *thermophilus, Streptococcus cristatus*, *Leuconostoc mesenteroides* subsp. *lactis*,

Enterococcus faecium, *Staphylococcus epidermidis* and *Staphylococcus hominis*. Presence of some LAB and staphylococci as observed in this research is in agreement with findings of Martin *et al*. (2003), Martin *et al*. (2007), Albesharat *et al*. (2011), Makino *et al*. (2011), Fernandez *et al*. (2013), Tusar *et al*. (2014) and Altuntus (2015).

8.4 Probiotic characterisation of identified LAB

Probiotics are living microorganisms that could be present in breast milk and other food products and when consumed in sufficient amount provide health benefit to host (FAO/WHO, 2001). Beneficial LAB play a vital role in competing favourably with pathogenic bacteria and excluding them in order to dominate the gut of babies (Jara *et al*., 2011 and Kavitha and Devasana 2013). For any beneficial bacterium to be classified as probiotic, it must meet the standards stipulated by Food and Agriculture Organisation and World Health Organisation (FAO/WHO, 2001). Some of the criteria include; viability, non-pathogenic, withstands harsh gastric conditions (acid and bile), produce antimicrobial substances that will inhibit or kill pathogens, adhere to gut epithelial tissues and bile salt hydrolase activity. Exopolysaccharide production and cholesterol assimilation are also important beneficial attributes of probiotic LAB (Jennifer *et al*. 2005, Shehata *et al*. 2016 and Yadav *et al*. 2016).

The selected LAB used for the probiotic studies in this research were representative of each genus and species of the eleven identified LAB (*Lactobacillus plantarum*, *Lactobacillus pentosus*, *Lactobacillus coryniformis*,

Lactobacillus fermentum, Leuconostoc mesenteroides subsp. *mesenteroides*, *Weissella confusa, Weissella paramesenteroides, Streptococcus salivarius* subsp. *thermophilus, Streptococcus cristatus, Leuconostoc mesenteroides* subsp. *lactis* and *Enterococcus faecium*). The probiotic assessments included; acid and bile resistance study, antimicrobial activity of LAB, bile salt hydrolase, cholesterol assimilation and exopolysaccharide production by LAB. Because *in vivo* studies are expensive, time consuming, needs stringent screening and approval by ethical committees, *in vitro* tests were used in this research.

8.4.1 Acid and bile resistance study

Acid and bile are very harsh substances present in the stomach and small intestine. Any potential beneficial bacteria must be able to tolerate and survive in low acid and bile environments (Pan *et al*. 2009). Ability of LAB to survive acid and bile facilitate colonisation in the gastrointestinal tract (GIT). Thus, allowing them to dominate and produce lactic acid and other antagonistic substances that annihilate pathogens (Hermanns *et al*. 2014).

Tolerance of LAB to pH 2.0, pH 3.0 and pH 4.0 with and without pepsin was examined in this research. Similarly, tolerance to 0.3%, 0.5%, 1% and 1.5% was also evaluated. The entire LAB could not withstand pH 2.0, but some especially *Lactobacillus* and *Streptococus* species were able to resist pH 2.0 with pepsin up to the third hour of the experiment. The pepsin therefore facilitated the survival of the LAB at this pH. Bendali *et al*. 2011 reported survival of *Lactobacillus* species in pH 2.0 with pepsin and this is in agreement with the research herein reported.

At pH 3.0 and pH 4.0 with and without pepsin, the entire LAB exhibited tolerance with the exception of *Leuconostoc mesenteroides* subsp. *mesenteroides* and *Weissella paramesenteroides* which did not survive in pH 3.0 without pepsin. Other researchers such as Hosseini *et al*. (2009), Zhang *et al*. (2013) and Hermanns *et al*. (2014) have reported protective effect of pepsin enzyme in promoting survivability of LAB to low pH. Moreover, tolerance of LAB to low pH is variable amongst species (Khalil *et al*. (2007).

Tolerance to bile salts is also vital in screening of beneficial bacteria with potential probiotic properties. The evaluated LAB in this research tolerated the critical 0.3% bile concentration standard which is a must for any potential beneficial bacteria to survive. Similarly, the screened LAB also tolerated higher concentrations of 0.5%, 1% and 1.5% bile salt.

8.4.2 Antimicrobial activity of LAB

Production of antagonistic substances by LAB such as lactic acid, hydrogen peroxide, bacteriocins and other antimicrobial substances are associated with potential probiotic benefits (Suskovic *et al*. 2010 and Leite *et al*. 2015). In fact, antimicrobial substances secreted by LAB have bacteriostatic and bactericidal effects on pathogenic bacteria (Pan *et al*. 2009 and Jara *et al*. 2011).

This study examined antimicrobial activity of LAB at sequential stages. It started with preliminary evaluation of production of antimicrobial substances using unbuffered and buffered agar spot tests (4.1.2 and 4.1.3). The entire LAB inhibited the growth of indicator bacteria (*Escherichia coli* NCTC 12900,

Salmonella Enteritidis DT124, *Staphylococcus aureus* CMCC 1930 and *Bacillus cereus* NCFB 13507). The cell free supernatant agar diffusion assay was relatively poor in inhibition of all pathogenic indicator bacteria. Except *Lactobacillus pentosus* and *Lactobacillus coryniformis* which inhibited growth of *Bacillus cereus, Escherichia coli* and *Salmonella* Enteritidis. But when cell free supernatant was concentrated using freeze dryer and diluted fivefold, increased inhibition of pathogens by some LAB was observed. *Lactobacillus pentosus, Lactobacillus coryniformis, Lactobacillus plantarum* and *Streptococcus cristatus* inhibited all the four indicator pathogenic bacteria. *Weissella confusa* inhibited *Bacillus cereus, Escherichia coli* and *Staphylococcus aureus* while *Lactobacillus fermentum* inhibited *Bacillus cereus* and *Escherichia coli.* Concentration of cell free supernatant increased potency of antimicrobial substances as observed in this study. Organic acid especially lactic acid could be the most antagonistic substance that inhibited some of the tested pathogens. The concentrated cell free supernatants of *Lactobacillus pentosus, Lactobacillus coryniformis, Lactobacillus plantarum* and *Streptococcus cristatus* that were heated at 63 ^{o}C for 30 min inhibited the growth of all four pathogens. This temperature/time combination was slightly higher than that used in pasteurisation (62.5 ^{o}C for 30 min) of human breast milk in milk banks (Kim and Unger 2010 and Haiden and Zeiglar, 2016). Similar trend was also reported by Al-Otaibi *et al.* (2012). Only *Streptococcus cristatus* was able to inhibit the four pathogens when the concentrated cell free supernatant was treated with enzymes (proteinase, protease and catalase). This could be as a result of production of bacteriocins and hydrogen peroxide.

Antimicrobial activity of LAB against *Bacillus cereus*, *Escherichia coli*, *Salmonella* Enteritidis and *Staphylococcus aureus* as observed in the agar spot test in this study is in agreement with Anyogu *et al*. (2014) and Leite *et al*. (2015) who studied antimicrobial activity of LAB from cassava and Brazillian kefir grains. The poor inhibition of pathogens by agar diffusion assay as observed in this study could be attributed to low concentration of antimicrobial substances as well as poor diffusion of supernatants. Al-Otaibi *et al*. (2012) also reported poor antimicrobial activity in agar well diffusion assay but strong antimicrobial activity in agar spot test.

8.4.3 Cholesterol assimilation, bile salt hydrolase and exopolysaccharide production by LAB

Beneficial LAB with probiotic properties are helpful in reducing incidence of infectious and non infectious illnesses such as diarrhoea, allergy and hyperlipedemia (Martin *et al*. 2003, Tusar *et al*. 2014 and Gunyatki and Asan-Ozusaglam 2018). Although, hypercholesterolemia is a rare incidence in infants, Yusuf *et al*. (2004) indicated high cholesterol and triglycerides (TG) in babies with family history of cardiovascular diseases. Deconjugation of bile salt by bile salt hydrolase enzyme is an important probiotic attribute that facilitate reduction of cholesterol (Damoddharan *et al*. 2015). Lactic acid bacterial exopolysaccharide is also associated with cholesterol reduction (Joshi and Koijan, 2014 and Panthavee *et al*. 2017). Exopolysaccharide facilitate movement of LAB from stomach to colon (Patil *et al*. 2015). It also aids in translocation of LAB from mother's gut to mammary gland (Jeurink *et al*. 2013).

This segment of research assessed production of exopolysaccharide by LAB (5.1.2), evaluated deconjugation of bile salt (bile salt hydrolase activity, 5.1.3) and examined cholesterol reduction potential (5.1.4) by the eleven selected LAB. The results obtained in this study indicated production of exopolysaccharide and bile salt hydrolase enzyme as well as assimilation of cholesterol by LAB. The findings of this research is in agreement with other researches such as Jiang *et al*. (2016) and Riaz-Rajoka *et al*. (2018) who reported production of exopolysaccharide by *Lactobacillus plantarum* and *Lactobacillus rhamnosus* isolated from human breast milk. Moreover, Annadraj and Sivasankar (2014) and Gunyatki and Asan-Ozusaglam (2018) reported cholesterol assimilation by *Lactobacillus oris* and *Lactobacillus gasseri* isolated from human breast milk. Additionally, Shehata *et al*. (2016) and Saraniya and Jeevaratnam (2015) reported bile salt hydrolase activity of some *Lactobacillus* species isolated from raw milk and fermented foods.

The positive effect of early colonisation of infants' gut by potentially probiotic lactic acid bacteria in human milk is an interesting and emerging area of research. Knowledge on probiotic attributes of LAB isolated from human breast milk is important in influencing lactating mothers and prospective mothers to breastfeed their babies diligently. Food and pharmaceutical industries could utilise information derived from this research on probiotic attributes of LAB in producing baby foods supplemented with probiotic bacteria. This could benefit babies that could not be breastfed due to communicable disease that could be passed on from mother to child. Overall, insight into probiotic attributes of LAB

will benefit health organisations, non-governmental agencies, researchers and the entire humanity.

8.5 Antimicrobial resistance of LAB and determination of resistance genes

When evaluating probiotic safety, two recommendations are presently acceptable. The European Food Safety Authority (EFSA) status of Qualified Presumption Safety (QPS) and the United State Food and Drug Administration (FDA) Generally Recognised as Safe (GRAS) (Sanders *et al*. 2010). Some LAB are regarded as safe (GRAS) by the United State FDA (Imperial and Albana, 2016) because of their ancient use in dairy and other fermented foods. But safety of some LAB could be at risk if they have potential of transferring antimicrobial resistance (AMR) genes (Sharma *et al*. 2017). Resistance of some LAB to antimicrobials has been attributed to indiscriminate application of antibiotics in food chain (Kabir *et al*. 2004, Adetunji, 2011, Zhang *et al*. 2011 and Mund *et al*. 2017). Misuse of antibiotics by humans including women of child bearing age is also associated with global AMR (Sapkota *et al*. 2010 and Jimenez *et al*. 2013). Moreover, vertical transfer of LAB with AMR genes from mother to infant through breast feeding is possible (Egervin *et al*. 2009, Rolain, 2013, Kozak *et al*. 2015 and Reis *et al*. 2016).

This study assessed phenotypic resistance of the eleven LAB to some antimicrobials. It also examined AMR genes that could be present in the LAB. Sensitivity of the eleven LAB to ciprofloxacin, gatifloxacin and quinupristin/dalfopristin was observed. Also, all LAB were sensitive to

ampicillin, penicillin and oxacillin with the exception of *Enterococcus faecium* that was resistant to ampicillin and oxacillin. The LAB were also sensitive to linezolid with the exception of *Lactobacillus fermentum*. However, most of the LAB were resistant to daptomycin, erythromycin, levofloxacin, tetracycline and vancomycin. Sensitivity of *Lactobacillus* species to ampicillin and penicillin as observed in this study is in agreement with other studies on human breast milk (Martin *et al*. 2005, Malek *et al*. 2010, Sharma *et al.* 2014, Kozak *et al*. 2015 and Sharma *et al.* 2017). Vancomycin resistance is very common amongst LAB (Gueomonde *et al*. 2013 and florez *et al*. 2016). Moreover, Kozak *et al*. (2015) and Sharma *et al.* (2017) reported vancomycin resistance in *Lactobacillus plantarum* and *Lactobacillus pentosus* isolated from human breast milk. Resistance of some LAB to clindamycin, ceftriaxone, erythromycin, gentamicin, levofloxacin and tetracycline as reported in this study is in agreement with other researchers (Ouoba *et al*. 2008, Kozak *et al*. 2015 and Sharma *et al*. 2017). In fact, erythromycin and tetracycline are constantly misused worldwide in human and food chain (Zhang *et al*. 2011 and Jimenez *et al*. 2013). Therefore, contribute to higher incidences of resistance of some LAB to these antimicrobials. In this study, resistance genes of *tet* (K), *tet* (L), *tet* (M) and *erm* (B) were confirmed for some of the LAB. The genes for *tet* (K), *tet* (L) and *tet* (M) were present in *Enterococcus faecium*, *tet* (L) in *Streptococcus cristatus* while *tet* (M) and *erm* (B) were present in *Streptococcus salivarius* subsp. *thermophilus*. None of the *Lactobacillus* species that were screened for resistance genes were confirmed although some showed positive amplicons. In contrast to this study, Egervin *et al*.

(2009) reported presence of *tet* (W) gene in *Lactobacillus plantarum* and *Lactobacillus reuteri* isolated from human breast milk. Other researchers have reported presence of *tet* (W), *tet* (O), *tet* (M) and *tet* (S) in *Lactobacillus* species isolated from fermented food products (Nawaz *et al*. 2011 and Thumma and Halami, 2012). According to FDA, presence of some *tet* genes in some probiotics supplemented in baby foods could not compromise clinical use of tetracycline, since tetracyclines are not recommended for children that are less than eight years old (Sanders *et al.* 2010). Some research on Human alpha-lactalbumin made lethal to tumour cells (HAMLET) a substance found in human breast milk is yielding promising results on its antimicrobial potentiating effect on staphylococci and numerous bacterial species with multi-drug resistance (Hakansson *et al*. 2011, Marks *et al*. 2012 and Marks *et al*. 2013). Therefore, presence of some tetracycline genes in *Enterococcus faecium*, *Streptococcus cristatus* and *Streptococcus salivarius* subsp. *thermophilus* as reported in this study is less likely to pose safety risks to babies.

8.6 Demographic study of human breast milk samples with relation to lactic acid bacterial population and diversity

Human breast milk is a source of nourishment for babies. Presence of some beneficial lactic acid bacteria in human breast milk is vital for intiation, maturation and development of babies' gut. Bifidobacteria, lactic acid bacteria and other commensal bacteria such as staphylococci have been isolated from human breast milk (Martin *et al*. 2009, Albesharat *et al*. 2011 and Sungaya *et al*. 2013). Babies suckling about 800 mg/day of breast milk will ingest about 10^5 to

10^7 bacterial cells (Martin *et al*. 2003 and Singh *et al*. 2014). However, differences have been reported to exist between gut microflora of breast fed babies to that of their formula fed counterparts (Martin *et al*. 2003 and Nasiraii *et al*. 2011). Gut microbiota of breast fed babies is composed of abundant Gram positive bacterial populations especially LAB (Khedid *et al*. 2009, Martin *et al*. 2009 and Kavitha and Devasena, 2015). Several factors could affect population and diversity of human breast milk microflora. Amongst the factors are ; gestational age, lactation stage, mode of delivery, geographical location, maternal nutritional status and diet (Mills *et al*. 2011, Nasiraii *et al*. 2011, Pardo *et al*. 2014 and Gomez-Gallego *et al*. 2016).

This study examined breast milk donors' demographic characteristics that could be related with composition of bacterial population and diversity in their breast milk samples. The data was collected using questionnare (Appendix 3) during sample collection. A population of 10^2 to 10^4 cfu/ml bacterial cells were enumerated in 18 out of the 20 breast milk samples. While a diversity of eleven LAB and two *Staphylococcus* species were identified. The population of enumerated bacteria in this study is in agreement with that of Perez *et al.* (2007) and Martin *et al*. (2009) who also enumerated a population of 10^2 to 10^4 cfu/ml. Diversity of *Staphylococcus, Lactobacillus, Bifidobacterium* and *Streptococcus* species were identified in Spanish (Collado *et al*. 2009), German and Austrian (Soto *et al*. 2014) human breast milk. Urbaniak *et al.* (2016) on the other hand reported diversity of *Staphylococcus,* Enterobacteriaceae and Pseudomonas species in Canadian human breast milk. Diversity of *Staphylococcus,*

Lactobacillus and *Streptococcus* species reported by Collado *et al*. (2009) and Soto *et al*. (2014) are in agreement with some of the diverse LAB from Nigerian human breast milk as reported in this study. There was no difference in the population and diversity of LAB with relation to sex of babies, lactation stage and number of donors' children. But maternal diet as observed in this study could affect diversity of LAB in human breast milk. Moreover, donors who consume complementary diet of rice, beans, stew and vegetable soup had diversity of two LAB with one sample having diversity of three LAB. In fact, diet has potential of modulating maternal intestinal microbiota (Gomez-Gallago *et al*. 2016) and consequently maternal gut bacteria translocate to mammary gland (Martin *et al*. 2003, Fernandez *et al*. 2004,Gueimonde*et al.*2007, Perez *et al*. 2007, Jimenez *et al.* 2008, Arroyo *et al.* 2010, Fernandenz *et al*. 2013 and Jeurink *et al*. 2013). Furthermore, Nasiraii *et al*. 2011 reported isolation of *Lactobacillus rhamnosus* LC705 from human breast milk samples of donors who were given probiotic diets containing *Lactobacillus rhamnosus* LC705.

8.7 Strengths and limitations of this research

Identification of lactic acid bacteria from human breast milk is imperative in knowing their diversity and potential probiotic properties that could be of immense benefit to babies. Some studies on microflora of human breast milk has been reported from countries such as Austria, Canada, Germany, Slovenia and Spain (Collado *et al*. 2009, Martin *et al*. 2012, Soto *et al*. 2014 and Tusar *et al*. 2014). But some differences were reported on composition of human breast milk which could be as a result of variation in dietry habits and other maternal factors

across different geographical areas. This research was therefore important because of the gaps in knowledge about diversity of LAB in Nigerian human breast milk and their potential probiotic properties. There is no information on antimicrobial resistance profile of LAB and antimicrobial resistance genes of LAB isolated from Nigerian human breast milk. There is also a gap in knowledge about factors that could contribute to population and diversity of LAB in human breast milk.

Standard and reliable laboratory techniques were used in this study. Substantial amount of isolates were recovered and a total of 108 were phenotypically examined. The genotypic techniques employed in this research consisted of repetitive sequence based PCR (rep-PCR) for grouping isolates and identification using16S rRNA gene sequencing. The probiotic studies have revealed the ability of the eleven screened LAB to tolerate acid and bile. They were able to produce antimicrobial substances that inhibited growth of some pathogenic bacteria. As well as produce exopolysaccharide and bile salt hydrolase enzyme, they also reduced cholesterol. These potential beneficial properties exhibited by the screened LAB could be of great benefit to breast fed babies' health and wellbeing. The antimicrobial resistance (AMR) study of the eleven screened LAB also used standard and reliable phenotypic and genotypic methods. The study has demonstrated the antimicrobial resistance profile of the studied LAB and some of their associated antimicrobial resistance genes. The AMR study has elucidated the global risks associated with misuse of antibiotics in human and food chain. Bacteria with AMR genes could be introduced into foods, thereby reaching

maternal gut, consequently transferred to mammary gland and eventually present in human breast milk.

In vitro methods were used to study LAB probiotic attributes in this research. *In vitro* assessment of probiotic characteristics are widely used in laboratory experiments because of accessibility and affordability (Pan *et al*. 2009, Bendali *et al*. 2011, Zhang *et al*. 2013 and Park and Liam, 2013). The complex issues and difficulties surrounding *in vivo* studies of probiotic bacteria resulted in the use of *in vitro* techniques. Although, *in vitro* methods are simulation of *in vivo* conditions, it also provides an estimation of what could happen *in vivo*. *In vitro* methods does not require meticulous screening and ethical approval, they are also less expensive and time consuming than *in vivo* studies. However, *in vivo* studies are important for confirmation of *in vitro* studies. This is especially vital when LAB with potential beneficial properties would be used in human clinical experiments.

Because of limited time frame for the research, a representative of each genus and species of the identified LAB were evaluated for probiotic and antimicrobial resistance studies. Each identified LAB should have been screened because probiotic attributes and antimicrobial resistance are strain specific (Begley *et al*. 2005 and Reis *et al*. 2016).

The number of donors was relatively small for the demographic study but the study has given an insight to some factors that could affect the diversity of LAB in human breast milk.

This research has revealed some of the lactic acid bacterial communities present in some samples of Nigerian human breast milk. It has explored the probiotic potential of some of the LAB and antimicrobial resistance of some of the LAB to antimicrobial substances. The research has therefore, contributed to the knowledge of microflora of some samples of Nigerian human breast milk. Additionally, it has contributed to the knowledge of the probiotic potentials of some of the lactic acid bacteria that have been investigated. The research has also given an insight into the antimicrobial resistance profile and antimicrobial resistance genes of some of the assessed LAB.

The antimicrobial resistance of some of the investigated LAB to some antimicrobials especially tetracycline as observed in this research could be attributed to misuse of antibiotics. Despite the resistance of some of the LAB investigated in this research to some antimicrobials, breast feeding is the best option for babies. In fact, human breast milk is nature's most balanced food for the nourishment of newly born babies as well as infants. Moreover, breast milk of healthy breast-feeding mothers is the best source of beneficial LAB for babies' gastrointestinal tracts (GIT). Breast fed infants have a greater diversity of LAB in their GITs than their formula-fed counterparts. Additionally, presence of large and diverse population of beneficial bacteria in the infant gut has been associated to good health in newly born babies and aids their healthy development in the future.

8.8 Recommendation for future studies

- *In vivo* studies on probiotic characteristics of the LAB to further confirm their beneficial properties.
- Investigation on mechanism of cholesterol assimilation by LAB is important.
- Studies on ability of the LAB to transfer antimicrobial resistance genes should be carried out to verify their safety for use as probiotic supplements.
- More extensive and collaborative research on relationship between breast milk donors' demography and population and diversity of LAB should be advocated.

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Appendix

Appendix 1: Participant information sheet

PARTICIPANT INFORMATION SHEET

Dear Participant

You are invited to take part in a research project entitled Enumeration, Isolation, Identification and Probiotic Characterisation of Lactic Acid Bacteria from Human Breast Milk.

But before you decide whether or not to take part, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Please feel free to ask questions if anything is unclear to you or if you would like more information.

N.B: For participants who cannot speak or read English, the investigator will translate and fill the form on their behalf.

The research is aimed at:

(1) Investigating the presence, type and population of some beneficial microorganisms in breast milk.
(2) Investigating some of the potential beneficial effects of the microorganisms.
(3) Investigating the possible relationship between the presence of the organisms in breast milk with donors' age, ethnicity, stage of lactation, number of children and diet.

What is the purpose of the study?

The purpose of this research is to find out if there are beneficial lactic acid bacteria in human breast milk. These microorganisms are important in the development of healthy digestive system of babies and therefore promote the health and well-being of babies.

Why have I been invited to participate?

You have been approached because you are a breast feeding mother.

Do I have to take part?

It is up to you to decide whether or not to take part, your participation is entirely voluntary. If you do decide to take part you will be given this information sheet to keep and you will be asked to sign the consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason**.** If you do decide to take part, you will be given this information sheet to keep and be asked to sign a consent form.

DURATION:

There will be 1 session only which will last only few minutes.

BENEFITS, RISK AND DISCOMFORT:

Your participation in this study will contribute to our understanding of the breast milk microbiota. There are no potential physical risks associated with participation in this experiment. Please note that your participation in this study will not embrace any particular benefit to you.

TASK REQUIREMENTS:

After reading and signing the consent form, you will be asked to express your breast milk in a sterile tube that will be provided.

ANONYMITY / CONFIDENTIALITY:

Every effort will be made to protect your privacy. Your name will not be used in any of the research reports or publications prepared with results obtained from this study. All information obtained in this study that identifies who you are will be recorded with a code number, and all studies are approved by ethical committee of Faculty of Life Science & Computing at London Metropolitan University**.** The information provided will be used for research purposes only.

RIGHT TO WITHDRAW:

Your participation in this experiment is completely voluntary.

You have the right to withdraw from this experiment at any time. If you feel anxious and/or uncomfortable at any stage of the study you could withdraw from the study just by letting the investigator know about your intention without any reasoning. Please bring your concerns to the researcher's attention immediately when and if needed.

If you decide to participate in this study, your participation and any information collected from you will be strictly confidential, and will be only available to the research team.

We would like to thank you, in advance, for your anticipated participation.

Supervisor: Dr. Hamid Ghoddusi

Supervisor's signature.....

Research Student: Binta Sambo Abdullahi

Research Student's signature.....

Participant name:

Participant's signature.....

Thanks Milking Moms!!!

We appreciate and value every drop of your donation

Appendix 2: Full consent form for breast milk collection

FULL CONSENT FORM FOR BREAST MILK COLLECTION

Title of project: Enumeration, Isolation, Identification and Probiotic Characterisation of Lactic Acid Bacteria from Human Breast Milk.

Name of volunteer (capitals).....

Please tick each statement to show your agreement

1. I confirm I have read the participant information sheet on the above project and have been given a copy to keep. I have had the opportunity to ask questions about the project and I am satisfied with the information that I have been given.

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason.

3. I agree to give breast milk samples for research in the above named project. I understand that all data and samples will be made anonymous prior to being circulated to other scientists. I give permission for a sub-sample of my breast milk to be transported to London Metropolitan Microbiology Research Unit in the United Kingdom for additional analyses.

4. I understand that I am giving the breast milk sample for the research study without any financial benefit.

5. I agree to donate my breast milk.

6. I agree to take part in the study and know how to contact the research team if I need to.

Volunteer's signature _____ Date _____

I confirm that I have fully explained the nature of this study to the above named volunteer.

Research student's signature _____ Date_____

Appendix 3: Breast milk donation form

Breast Milk Donation

DONOR REGISTRATION FORM

SAMPLE CODE:

Date _____

Name _____ Age _____ DOB _____

Phone (Home) _____ Work _____ Other _____

Address _____

City _____ State _____ Zip _____

Occupation _____

Baby's Name _____ Sex _____ DOB _____

Birth Weight _____ Height _____ Last height / weight _____ Date _____

Was baby full term? _____ if no, what was the gestational age? _____

Ethnic background?

Number of children including the newborn?

Typical daily food of mum?

Do you have any fever with headache? Yes _____ No _____If yes, please explain:

_____

_____

Appendix 4: Results of acid and bile resistance study of lactic acid bacteria isolated from human breast milk

Table 3.1: Acid tolerance of *Leuconostoc mesenteroides* subsp. *mesenteroides*.

Time (min)	Control[a] (cfu/ml)	pH2 (cfu/ml)	SR[b] (%)	pH3 (cfu/ml)	SR[b] (%)	pH4 (cfu/ml)	SR[b] (%)	pH2+ pepsin (cfu/ml)	SR[b] (%)	pH3+ pepsin (cfu/ml)	SR[b] (%)	pH4+ pepsin (cfu/ml)	SR[b] (%)
0	7.99±0.20	<10	0.0	8.40±0.29	105	8.46±0.14	105	<10	0.0	8.56±0.27	107	8.59±0.26	107
30	7.85±0.11	<10	0.0	8.11±0.16	103	8.41±0.21	107	<10	0.0	8.54±0.12	108	8.63±0.22	109
60	7.91±0.32	<10	0.0	0.00	0.0	7.90±0.19	99	<10	0.0	6.86±0.10	86	7.77±0.10	98
90	8.57±0.26	<10	0.0	0.00	0.0	7.92±0.11	92	<10	0.0	6.72±0.18	78	7.81±0.14	91
120	8.41±0.17	<10	0.0	0.00	0.0	7.69±0.27	91	<10	0.0	6.56±0.33	77	7.67±0.17	91
150	8.62±0.38	<10	0.0	0.00	0.0	7.80±0.13	90	<10	0.0	6.48±0.29	75	7.82±0.22	90
180	8.60±0.13	<10	0.0	0.00	0.0	7.82±0.16	90	<10	0.0	6.40±0.13	74	7.80±0.13	90

Table 3.2: Bile salt tolerance of *Leuconostoc mesenteroides* subsp. *mesenteroides*.

Time (min)	Control[a] (cfu/ml)	0.3% bile salt	SR (%)[b]	0.5% bile salt	SR (%)[b]	1% bile salt	SR (%)[b]	1.5% bile salt	SR (%)[b]
0	7.99±0.20	7.70±0.20	96	7.69±0.24	96	7.67±0.18	96	7.60±0.21	95
30	7.85±0.11	7.81±0.15	99	7.79±0.16	99	7.72±0.29	98	7.70±0.27	98
60	7.91±0.32	7.81±0.21	98	7.81±0.20	98	7.74±0.25	97	7.71±0.16	97
90	8.57±0.26	7.74±0.23	90	7.63±0.25	89	6.91±0.15	80	6.90±0.22	80
120	8.41±0.17	7.57±0.14	89	7.57±0.19	89	6.70±0.12	79	6.60±0.20	78
150	8.62±0.38	7.68±0.11	89	7.46±0.17	86	5.68±0.24	65	5.57±0.16	64
180	8.60±0.13	7.40±0.21	86	6.30±0.23	73	5.15±0.30	59	5.11±0.13	59

Control, pH and bile salt values are mean $\log_{10}$ (cfu/ml) ± standard deviation of duplicate experiments

a = control cells in pH 7.3 (phosphate buffered saline)

b = SR (survival ratio), stressed cell number divided by control cell number multiplied by 100

Table 3.3: Acid tolerance of *Weissella paramesenteroides.*

Time (min)	Control[a] (cfu/ml)	pH2 (cfu/ml)	SR[b] (%)	pH3 (cfu/ml)	SR[b] (%)	pH4 (cfu/ml)	SR[b] (%)	pH2+ pepsin (cfu/ml)	SR[b] (%)	pH3+ pepsin (cfu/ml)	SR[b] (%)	pH4+ pepsin (cfu/ml)	SR[b] (%)
0	8.39±0.24	<10	0.0	7.90±0.18	94	8.74±0.22	104	<10	0.0	7.76±0.17	92	8.39±0.14	100
30	8.49±0.21	<10	0.0	5.90±0.10	69	8.50±0.13	100	<10	0.0	6.98±0.31	82	8.46±0.27	99
60	8.69±0.15	<10	0.0	0.00	0.0	8.00±0.26	92	<10	0.0	7.00±0.19	80	7.96±0.23	91
90	8.99±0.28	<10	0.0	0.00	0.0	8.00±0.26	88	<10	0.0	7.00±0.19	77	8.00±0.26	88
120	9.00±0.11	<10	0.0	0.00	0.0	8.00±0.26	88	<10	0.0	7.00±0.19	77	8.00±0.26	88
150	9.04±0.16	<10	0.0	0.00	0.0	8.00±0.26	88	<10	0.0	6.95±0.25	76	8.00±0.26	88
180	9.07±0.10	<10	0.0	0.00	0.0	8.00±0.26	88	<10	0.0	6.91±0.21	76	8.00±0.26	88

Table 3.4: Bile salt tolerance of *Weissella paramesenteroides.*

Time (min)	Control[a] (cfu/ml)	0.3%bile salt	SR (%)[b]	0.5%bile salt	SR (%)[b]	1% bile salt	SR (%)[b]	1.5%bile salt	SR (%)[b]
0	8.39±0.24	7.92±0.12	94	7.85±0.29	93	7.81±0.15	93	7.81±0.19	93
30	8.49±0.21	7.98±0.16	94	7.95±0.24	93	7.93±0.22	93	7.82±0.11	92
60	8.69±0.15	8.04±0.11	92	8.00±0.26	92	7.98±0.17	91	7.98±0.16	91
90	8.99±0.28	8.00±0.26	88	7.98±0.10	88	7.96±0.30	88	7.95±0.23	88
120	9.00±0.11	8.00±0.26	88	7.96±0.21	88	7.90±0.14	87	7.83±0.21	87
150	9.04±0.16	7.96±0.10	88	7.88±0.24	87	7.74±0.30	85	7.44±0.16	82
180	9.07±0.10	7.70±0.13	84	7.67±0.18	84	7.50±0.12	82	7.27±0.10	80

Control, pH and bile salt values are mean $\log_{10}$ (cfu/ml) ± standard deviation of duplicate experiments

a = control cells in pH 7.3 (phosphate buffered saline)

b = SR (survival ratio), stressed cell number divided by control cell number multiplied by 100

Table 3.5: Acid tolerance of *Lactobacillus fermentum.*

Time (min)	Control[a] (cfu/ml)	pH2 (cfu/ml)	SR[b] (%)	pH3 (cfu/ml)	SR[b] (%)	pH4 (cfu/ml)	SR[b] (%)	pH2+ pepsin (cfu/ml)	SR[b] (%)	pH3+ pepsin (cfu/ml)	SR[b] (%)	pH4+ pepsin (cfu/ml)	SR[b] (%)
0	8.73±0.18	4.82±0.32	55	7.96±0.23	91	8.96±0.12	102	4.90±0.30	56	7.98±0.25	91	8.90±0.14	102
30	8.80±0.20	4.79±0.30	54	7.93±0.11	90	8.91±0.15	101	4.87±0.23	55	7.96±0.18	90	8.90±0.14	101
60	8.75±0.31	4.41±0.27	50	6.97±0.16	79	8.92±0.22	102	4.77±0.27	54	7.98±0.13	91	8.74±0.10	100
90	8.83±0.26	<10	0.0	7.00±0.19	79	8.93±0.11	101	4.74±0.15	53	7.99±0.34	90	8.91±0.19	100
120	8.89±0.13	<10	0.0	6.99±0.16	78	8.96±0.19	100	4.59±0.22	51	8.00±0.32	90	8.95±0.24	100
150	8.94±0.20	<10	0.0	6.97±0.18	78	7.99±0.17	89	4.47±0.27	50	7.00±0.28	78	8.97±0.12	100
180	9.00±0.23	<10	0.0	6.97±0.21	77	8.00±0.11	88	4.23±0.16	47	7.00±0.28	77	8.00±0.19	88

Table 3.6: Bile salt tolerance of *Lactobacillus fermentum.*

Time (min)	Control[a] (cfu/ml)	0.3%bile salt	SR (%)[b]	0.5%bile salt	SR (%)[b]	1% bile salt	SR (%)[b]	1.5%bile salt	SR (%)[b]
0	8.73±0.18	8.00±0.25	91	8.00±0.13	91	7.99±0.27	91	7.97±0.32	91
30	8.80±0.20	7.99±0.36	90	7.98±0.17	90	7.97±0.23	90	7.97±0.32	90
60	8.75±0.31	8.00±0.25	91	7.99±0.26	91	7.94±0.16	90	7.91±0.30	90
90	8.83±0.26	8.00±0.25	90	8.00±0.13	90	7.90±0.12	89	7.90±0.27	89
120	8.89±0.13	7.99±0.36	89	7.99±0.18	89	7.28±0.11	81	7.26±0.17	81
150	8.94±0.20	7.00±0.22	78	7.00±0.11	78	5.38±0.16	60	5.18±0.21	57
180	9.00±0.23	6.98±0.17	77	6.00±0.16	66	<10	0.0	<10	0.0

Control, pH and bile salt values are mean $\log_{10}$ (cfu/ml) ± standard deviation of duplicate experiments

a = control cells in pH 7.3 (phosphate buffered saline)

b = SR (survival ratio), stressed cell number divided by control cell number multiplied by 100

Table 3.7: Acid tolerance of *Lactobacillus pentosus.*

Time (min)	Control[a] (cfu/ml)	pH2 (cfu/ml)	SR[b] (%)	pH3 (cfu/ml)	SR[b] (%)	pH4 (cfu/ml)	SR[b] (%)	pH2+ pepsin (cfu/ml)	SR[b] (%)	pH3+ pepsin (cfu/ml)	SR[b] (%)	pH4+ pepsin (cfu/ml)	SR[b] (%)
0	7.85±0.37	7.59±0.27	96	7.77±0.41	98	7.83±0.11	99	7.63±0.16	97	7.84±0.52	99	7.91±0.24	100
30	7.83±0.29	<10	0.0	7.77±0.41	99	7.86±0.26	100	6.94±0.10	88	7.86±0.44	100	7.92±0.30	101
60	7.90±0.28	<10	0.0	7.76±0.27	98	7.92±0.30	100	6.89±0.21	87	7.89±0.20	99	7.94±0.28	100
90	8.65±0.15	<10	0.0	7.79±0.33	90	7.68±0.19	88	5.46±0.17	63	7.91±0.20	91	8.65±0.17	100
120	8.67±0.43	<10	0.0	7.82±0.18	90	8.67±0.11	100	4.11±0.20	47	7.92±0.37	91	8.69±0.13	100
150	8.70±0.16	<10	0.0	7.83±0.22	89	8.69±0.14	99	3.30±0.11	37	7.89±0.16	90	8.69±0.30	99
180	8.70±0.22	<10	0.0	7.77±0.15	89	8.71±0.19	100	3.14±0.17	36	7.91±0.21	90	8.74±0.14	100

Table 3.8: Bile salt tolerance of *Lactobacillus pentosus.*

Time (min)	Control[a] (cfu/ml)	0.3% bile salt	SR (%)[b]	0.5% bile salt	SR (%)[b]	1% bile salt	SR (%)[b]	1.5% bile salt	SR (%)[b]
0	7.85±0.37	7.90±0.66	100	7.87±0.12	100	7.86±0.45	100	7.85±0.13	100
30	7.83±0.29	7.93±0.23	101	7.90±0.21	100	7.89±0.38	100	7.89±0.11	100
60	7.90±0.28	7.95±0.27	100	7.91±0.25	100	7.89±0.38	99	7.87±0.16	99
90	8.65±0.15	7.97±0.29	92	7.93±0.12	91	7.91±0.24	91	7.90±0.20	91
120	8.67±0.43	8.00±0.18	92	7.96±0.34	91	7.94±0.30	91	7.92±0.25	91
150	8.70±0.16	7.98±0.23	91	7.95±0.28	91	7.91±0.19	90	7.88±0.16	90
180	8.70±0.22	7.99±0.15	91	7.95±0.16	91	7.93±0.21	91	7.92±0.10	91

Control, pH and bile salt values are mean $\log_{10}$ (cfu/ml) ± standard deviation of duplicate experiments

a = control cells in pH 7.3 (phosphate buffered saline)

b = SR (survival ratio), stressed cell number divided by control cell number multiplied by 100

Table 3.9: Acid tolerance of *Weissella confusa.*

Time (min)	Control[a] (cfu/ml)	pH2 (cfu/ml)	SR[b] (%)	pH3 (cfu/ml)	SR[b] (%)	pH4 (cfu/ml)	SR[b] (%)	pH2+ pepsin (cfu/ml)	SR[b](%)	pH3+ pepsin (cfu/ml)	SR[b] (%)	pH4+ pepsin (cfu/ml)	SR[b] (%)
0	7.68±0.71	<10	0.0	7.66±0.27	99	7.77±0.16	101	<10	0.0	7.49±0.33	97	7.78±0.20	101
30	7.74±0.52	<10	0.0	7.59±0.17	97	7.79±0.24	100	<10	0.0	7.63±0.38	98	7.80±0.23	100
60	7.78±0.23	<10	0.0	7.56±0.10	97	7.81±0.11	100	<10	0.0	7.73±0.26	99	7.81±0.15	100
90	7.83±0.25	<10	0.0	7.39±0.19	94	7.85±0.18	100	<10	0.0	7.79±0.30	99	7.86±0.15	100
120	7.88±0.20	<10	0.0	7.23±0.11	91	7.89±0.24	100	<10	0.0	7.82±0.30	99	7.89±0.17	100
150	7.89±0.34	<10	0.0	7.20±0.11	91	7.91±0.29	100	<10	0.0	7.72±0.21	97	7.94±0.11	100
180	7.91±0.23	<10	0.0	5.11±0.20	64	7.93±0.20	100	<10	0.0	7.53±0.24	95	7.97±0.11	100

Table 3.10: Bile salt tolerance of *Weissella confusa.*

Time (min)	Control[a] (cfu/ml)	0.3% bile salt	SR (%)[b]	0.5% bile salt	SR (%)[b]	1% bile salt	SR (%)[b]	1.5% bile salt	SR (%)[b]
0	7.68±0.71	7.74±0.29	100	7.72±0.62	100	7.62±0.30	99	7.61±0.13	99
30	7.74±0.52	7.75±0.36	100	7.73±0.40	99	7.71±0.15	99	7.71±0.17	99
60	7.78±0.23	7.74±0.24	99	7.71±0.23	99	7.70±0.20	99	7.61±0.13	97
90	7.83±0.25	7.20±0.21	91	7.17±0.32	91	6.14±0.20	78	6.07±0.18	77
120	7.88±0.20	6.27±0.18	79	6.23±0.27	79	5.14±0.22	65	5.07±0.12	64
150	7.89±0.34	6.14±0.26	77	6.07±0.17	76	4.07±0.10	51	<10	0.0
180	7.91±0.23	5.32±0.28	67	5.17±0.22	65	<10	0.0	<10	0.0

Control, pH and bile salt values are mean $\log_{10}$ (cfu/ml) ± standard deviation of duplicate experiments

a = control cells in pH 7.3 (phosphate buffered saline)

b = SR (survival ratio), stressed cell number divided by control cell number multiplied by 100

Table 3.11: Acid tolerance of *Streptococcus salivarius* subsp. *thermophilus*.

Time (min)	Control[a] (cfu/ml)	pH2 (cfu/ml)	SR[b] (%)	pH3 (cfu/ml)	SR[b] (%)	pH4 (cfu/ml)	SR[b] (%)	pH2+ pepsin (cfu/ml)	SR[b] (%)	pH3+ pepsin (cfu/ml)	SR[b] (%)	pH4+ pepsin (cfu/ml)	SR[b] (%)
0	7.78±0.32	<10	0.00	7.54±0.19	96	7.62±0.24	97	3.56±0.11	45	7.53±0.44	96	7.69±0.55	98
30	7.75±0.37	<10	0.00	7.57±0.12	97	7.73±0.30	99	3.49±0.14	45	7.44±0.37	96	7.78±0.38	100
60	7.81±0.25	<10	0.00	7.43±0.21	95	7.78±0.26	99	3.36±0.10	43	7.47±0.41	95	7.82±0.22	100
90	7.85±0.17	<10	0.00	7.32±0.20	93	7.86±0.21	100	<10	0.00	7.36±0.30	93	7.88±0.31	100
120	8.60±0.41	<10	0.00	6.59±0.14	76	8.60±0.18	100	<10	0.00	6.66±0.30	77	8.63±0.26	100
150	8.65±0.34	<10	0.00	6.20±0.13	71	8.69±0.21	100	<10	0.00	6.61±0.28	76	8.70±0.17	100
180	8.71±0.23	<10	0.00	5.17±0.10	59	8.72±0.25	100	<10	0.00	6.55±0.22	75	8.74±0.20	100

Table 3.12: Bile salt tolerance of *Streptococcus salivarius* subsp. *thermophilus*.

Time (min)	Control[a] (cfu/ml)	0.3% bile salt	SR (%)[b]	0.5% bile salt	SR (%)[b]	1% bile salt	SR (%)[b]	1.5% bile salt	SR (%)[b]
0	7.78±0.32	7.61±0.14	97	7.56±0.28	97	7.51±0.20	96	7.46±0.15	95
30	7.75±0.37	7.62±0.22	98	7.57±0.25	97	7.44±0.28	96	7.32±0.21	94
60	7.81±0.25	7.44±0.18	95	7.36±0.21	94	7.32±0.34	93	7.27±0.20	93
90	7.85±0.17	7.25±0.11	92	7.14±0.26	91	6.59±0.17	83	6.53±0.26	83
120	8.60±0.41	6.62±0.15	76	6.51±0.15	75	6.50±0.20	75	5.39±0.19	0.0
150	8.65±0.34	5.53±0.19	63	5.47±0.23	63	5.34±0.16	61	<10	0.0
180	8.71±0.23	5.44±0.11	62	5.20±0.23	59	5.07±0.11	58	<10	0.0

Control, pH and bile salt values are mean $\log_{10}$ (cfu/ml) ± standard deviation of duplicate experiments

a = control cells in pH 7.3 (phosphate buffered saline)

b = SR (survival ratio), stressed cell number divided by control cell number multiplied by 100

Table 3.13: Acid tolerance of *Lactobacillus plantarum.*

Time (min)	Control[a] (cfu/ml)	pH2 (cfu/ml)	SR[b] (%)	pH3 (cfu/ml)	SR[b] (%)	pH4 (cfu/ml)	SR[b] (%)	pH2+ pepsin (cfu/ml)	SR[b] (%)	pH3+ pepsin (cfu/ml)	SR[b] (%)	pH4+ pepsin (cfu/ml)	SR[b] (%)
0	7.70±0.68	6.21±0.19	80	7.68±0.40	100	7.72±0.26	100	6.90±0.13	89	7.69±0.41	99	7.74±0.29	100
30	7.76±0.43	<10	0.0	7.72±0.33	100	7.77±0.34	100	6.92±0.13	89	7.74±0.34	99	7.78±0.36	100
60	7.79±0.47	<10	0.0	7.77±0.35	100	7.82±0.25	100	6.89±0.17	88	7.78±0.20	99	7.85±0.30	100
90	7.85±0.51	<10	0.0	7.80±0.22	98	7.86±0.25	100	6.82±0.14	86	7.81±0.29	99	7.87±0.34	100
120	7.89±0.29	<10	0.0	7.73±0.27	97	7.92±0.31	100	4.68±0.19	59	7.82±0.29	99	7.93±0.41	100
150	7.96±0.33	<10	0.0	7.69±0.20	96	7.96±0.30	100	4.59±0.11	57	7.79±0.24	97	7.96±0.43	100
180	7.99±0.55	<10	0.0	7.65±0.20	95	8.00±0.18	100	4.38±0.11	54	7.74±0.20	96	8.00±0.32	100

Table 3.14: Bile salt tolerance of *Lactobacillus plantarum.*

Time (min)	Control[a] (cfu/ml)	0.3% bile salt	SR (%)[b]	0.5% bile salt	SR (%)[b]	1% bile salt	SR (%)[b]	1.5% bile salt	SR (%)[b]
0	7.70±0.68	7.69±0.54	99	7.68±0.48	99	7.61±0.21	98	7.56±0.30	98
30	7.76±0.43	7.74±0.47	99	7.71±0.23	99	7.68±0.26	98	7.65±0.34	98
60	7.79±0.47	7.77±0.31	99	7.74±0.15	99	7.71±0.17	99	7.68±0.34	98
90	7.85±0.51	7.82±0.35	99	7.79±0.25	99	7.74±0.24	98	7.71±0.23	98
120	7.89±0.29	7.77±0.22	98	7.74±0.15	98	7.68±0.13	97	7.65±0.31	96
150	7.96±0.33	7.66±0.18	96	7.63±0.20	95	7.56±0.19	95	7.55±0.25	94
180	7.99±0.55	7.60±0.31	95	7.47±0.11	93	7.36±0.16	92	7.27±0.22	91

Control, pH and bile salt values are mean $\log_{10}$ (cfu/ml) ± standard deviation of duplicate experiments

a = control cells in pH 7.3 (phosphate buffered saline)

b = SR (survival ratio), stressed cell number divided by control cell number multiplied by 100

Table 3.15: Acid tolerance of *Lactobacillus coryniformis*.

Time (min)	Control[a] (cfu/ml)	pH2 (cfu/ml)	SR[b] (%)	pH3 (cfu/ml)	SR[b] (%)	pH4 (cfu/ml)	SR[b] (%)	pH2+ pepsin (cfu/ml)	SR[b](%)	pH3+ pepsin (cfu/ml)	SR[b] (%)	pH4+ pepsin (cfu/ml)	SR[b] (%)
0	7.86±0.50	4.34±0.11	55	7.72±0.23	98	7.85±0.40	99	4.49±0.16	57	7.80±0.36	99	7.87±0.67	100
30	7.93±0.59	3.27±0.10	41	7.76±0.21	97	7.91±0.27	99	4.38±0.21	55	7.77±0.31	97	7.92±0.73	99
60	7.97±0.46	3.23±0.15	40	7.78±0.21	97	7.94±0.21	99	3.46±0.12	43	7.85±0.39	98	7.98±0.77	100
90	8.73±0.51	3.11±0.18	35	7.66±0.17	87	8.72±0.33	99	3.23±0.10	36	7.87±0.26	90	8.74±0.59	100
120	8.79±0.51	<10	0.0	7.70±0.26	87	8.74±0.31	99	<10	0.0	7.92±0.39	90	8.80±0.34	100
150	8.86±0.32	<10	0.0	7.83±0.14	88	8.78±0.38	99	<10	0.0	7.95±0.22	89	8.87±0.31	100
180	8.89±0.24	<10	0.0	7.93±0.29	89	8.79±0.38	98	<10	0.0	7.97±0.27	89	8.90±0.31	100

Table 3.16: Bile salt tolerance of *Lactobacillus coryniformis*

Time (min)	Control[a] (cfu/ml)	0.3% bile salt	SR (%)[b]	0.5% bile salt	SR (%)[b]	1% bile salt	SR (%)[b]	1.5% bile salt	SR (%)[b]
0	7.86±0.50	7.85±0.18	99	7.76±0.25	98	7.67±0.36	97	7.65±0.22	97
30	7.93±0.59	7.86±0.15	99	7.76±0.21	97	7.74±0.28	97	7.73±0.17	97
60	7.97±0.46	7.86±0.15	98	7.77±0.19	97	7.71±0.21	96	7.70±0.29	96
90	8.73±0.51	7.60±0.23	87	7.57±0.23	86	7.55±0.30	86	7.53±0.34	86
120	8.79±0.51	7.59±0.13	86	7.54±0.23	85	7.51±0.16	85	7.49±0.16	85
150	8.86±0.32	7.57±0.17	85	7.51±0.17	84	7.50±0.16	84	7.44±0.20	83
180	8.89±0.24	7.54±0.14	84	7.47±0.13	84	7.46±0.11	83	7.38±0.13	82

Control, pH and bile salt values are mean $\log_{10}$ (cfu/ml) ± standard deviation of duplicate experiments

a = control cells in pH 7.3 (phosphate buffered saline)

b = SR (survival ratio), stressed cell number divided by control cell number multiplied by 100

Table 3.17: Acid tolerance of *Streptococcus cristatus.*

Time (min)	Control[a] (cfu/ml)	pH2 (cfu/ml)	SR[b] (%)	pH3 (cfu/ml)	SR[b] (%)	pH4 (cfu/ml)	SR[b] (%)	pH2+ pepsin (cfu/ml)	SR[b](%)	pH3+ pepsin (cfu/ml)	SR[b] (%)	pH4+ pepsin (cfu/ml)	SR[b] (%)
0	8.74±0.77	6.96±0.14	79	8.56±0.24	97	8.78±0.32	100	6.97±0.20	79	8.62±0.63	98	8.80±0.34	100
30	8.78±0.65	3.14±0.10	35	8.62±0.36	98	8.79±0.35	100	6.17±0.16	70	8.66±0.28	98	8.83±0.28	100
60	8.81±0.65	<10	0.0	8.69±0.21	98	8.82±0.27	100	5.54±0.21	62	8.73±0.42	99	8.84±0.22	100
90	8.85±0.40	<10	0.0	8.71±0.17	98	8.86±0.24	100	5.53±0.21	62	8.77±0.47	99	8.86±0.13	100
120	8.88±0.32	<10	0.0	8.69±0.30	97	8.88±0.14	100	5.41±0.13	60	8.81±0.36	99	8.88±0.20	100
150	8.89±0.32	<10	0.0	8.64±0.18	97	8.90±0.14	100	4.27±0.19	48	8.84±0.24	99	8.92±0.15	100
180	8.93±0.27	<10	0.0	8.49±0.20	94	8.94±0.21	100	3.34±0.10	37	8.88±0.30	99	8.95±0.20	100

Table 3.18: Bile salt tolerance of *Streptococcus cristatus*

Time (min)	Control[a] (cfu/ml)	0.3% bile salt	SR (%)[b]	0.5% bile salt	SR (%)[b]	1% bile salt	SR (%)[b]	1.5% bile salt	SR (%)[b]
0	8.74±0.77	8.68±0.56	99	8.67±0.12	99	8.61±0.21	98	8.53±0.15	97
30	8.78±0.65	8.71±0.41	99	8.69±0.17	99	8.67±0.38	98	8.61±0.26	98
60	8.81±0.65	8.74±0.47	99	8.72±0.24	98	8.70±0.32	98	8.64±0.32	98
90	8.85±0.40	8.79±0.32	99	8.74±0.29	98	8.72±0.32	98	8.69±0.18	98
120	8.88±0.32	8.75±0.25	98	8.71±0.14	98	8.66±0.24	97	8.63±0.23	97
150	8.89±0.32	8.72±0.30	98	8.68±0.20	97	8.61±0.27	96	8.54±0.20	96
180	8.93±0.27	8.60±0.22	96	8.56±0.23	95	8.49±0.28	94	8.25±0.20	92

Control, pH and bile salt values are mean $\log_{10}$ (cfu/ml) ± standard deviation of duplicate experiments

a = control cells in pH 7.3 (phosphate buffered saline)

b = SR (survival ratio), stressed cell number divided by control cell number multiplied by 100

Table 3.19: Acid tolerance of *Leuconostoc mesenteroides* subsp. *lactis*.

Time (min)	Control[a] (cfu/ml)	pH2 (cfu/ml)	SR[b] (%)	pH3 (cfu/ml)	SR[b] (%)	pH4 (cfu/ml)	SR[b] (%)	pH2+ pepsin (cfu/ml)	SR[b] (%)	pH3+ pepsin (cfu/ml)	SR[b] (%)	pH4+ pepsin (cfu/ml)	SR[b] (%)
0	8.70±0.82	6.34±0.14	72	8.63±0.28	99	8.74±0.17	100	7.79±0.15	89	8.69±0.43	99	8.75±0.35	100
30	8.75±0.87	3.20±0.20	36	8.66±0.31	98	8.77±0.22	100	7.74±0.11	88	8.69±0.43	99	8.78±0.50	100
60	8.80±0.61	<10	0.0	8.70±0.37	98	8.81±0.27	100	6.79±0.20	77	8.74±0.47	99	8.81±0.53	100
90	8.83±0.33	<10	0.0	8.75±0.22	99	8.84±0.19	100	6.65±0.20	75	8.81±0.50	99	8.85±0.27	100
120	8.86±0.39	<10	0.0	8.71±0.16	98	8.86±0.19	100	3.51±0.10	39	8.76±0.31	98	8.87±0.19	100
150	8.88±0.44	<10	0.0	8.57±0.25	96	8.90±0.25	100	3.11±0.18	35	8.72±0.28	98	8.91±0.24	100
180	8.92±0.48	<10	0.0	8.47±0.23	94	8.93±0.35	100	<10	0.0	8.68±0.35	97	8.93±0.30	100

Table 3.20: Bile salt tolerance of *Leuconostoc mesenteroides* subsp. *lactis*.

Time (min)	Control[a] (cfu/ml)	0.3% bile salt	SR (%)[b]	0.5% bile salt	SR (%)[b]	1% bile salt	SR (%)[b]	1.5% bile salt	SR (%)[b]
0	8.70±0.82	8.61±0.17	98	8.57±0.33	98	8.55±0.20	98	8.54±0.39	98
30	8.75±0.87	8.69±0.22	99	8.65±0.46	98	8.62±0.32	98	8.59±0.20	98
60	8.80±0.61	8.71±0.25	98	8.69±0.21	98	8.65±0.18	98	8.64±0.26	98
90	8.83±0.33	8.74±0.20	98	8.72±0.40	98	8.68±0.13	98	8.66±0.43	98
120	8.86±0.39	8.69±0.14	98	8.65±0.15	97	8.64±0.36	97	8.62±0.30	97
150	8.88±0.44	8.53±0.11	96	8.51±0.22	95	8.49±0.30	95	8.47±0.21	95
180	8.92±0.48	8.49±0.21	95	8.44±0.31	94	8.34±0.16	93	8.30±0.18	92

Control, pH and bile salt values are mean $\log_{10}$ (cfu/ml) ± standard deviation of duplicate experiments

a = control cells in pH 7.3 (phosphate buffered saline)

b = SR (survival ratio), stressed cell number divided by control cell number multiplied by 100

Table 3.21: Acid tolerance of *Enterococcus faecium*

Time (min/hour)	Control[a] (cfu/ml)	pH2 (cfu/ml)	SR[b] (%)	pH3 (cfu/ml)	SR[b] (%)	pH4 (cfu/ml)	SR[b] (%)	pH2+ pepsin (cfu/ml)	SR[b] (%)	pH3+ pepsin (cfu/ml)	SR[b] (%)	pH4+ pepsin (cfu/ml)	SR[b] (%)
0	7.83±0.44	7.53±0.12	96	7.72±0.24	98	7.85±0.52	100	7.64±0.13	97	7.79±0.37	99	7.85±0.35	100
30	7.86±0.23	6.54±0.19	83	7.74±0.20	98	7.86±0.41	100	6.70±0.10	85	7.81±0.30	99	7.86±0.35	100
60	7.88±0.50	4.69±0.10	59	7.76±0.35	98	7.87±0.39	100	4.77±0.16	60	7.82±0.22	99	7.88±0.28	100
90	7.89±0.27	<10	0.0	7.71±0.44	97	7.89±0.40	100	4.54±0.16	57	7.84±0.42	99	7.90±0.20	100
120	7.92±0.31	<10	0.0	7.69±0.20	97	7.92±0.46	100	3.46±0.22	43	7.83±0.31	98	7.94±0.39	100
150	7.94±0.26	<10	0.0	7.69±0.18	96	7.94±0.32	100	3.07±0.17	38	7.77±0.25	97	7.95±0.47	100
180	7.95±0.31	<10	0.0	7.65±0.27	96	7.96±0.28	100	<10	0.0	7.67±0.25	96	7.97±0.31	100

Table 3.22: Bile salt tolerance of *Enterococcus faecium*

Time (min)	Control[a] (cfu/ml)	0.3% bile salt	SR (%)[b]	0.5% bile salt	SR (%)[b]	1% bile salt	SR (%)[b]	1.5% bile salt	SR (%)[b]
0	7.83±0.44	7.71±0.63	98	7.69±0.27	98	7.69±0.53	98	7.68±0.17	98
30	7.86±0.23	7.77±0.46	98	7.74±0.15	98	7.72±0.41	98	7.70±0.20	98
60	7.88±0.50	7.78±0.35	98	7.77±0.23	98	7.74±0.24	98	7.74±0.23	98
90	7.89±0.27	7.74±0.23	98	7.74±0.27	98	7.72±0.19	97	7.69±0.32	97
120	7.92±0.31	7.72±0.41	97	7.70±0.32	97	7.67±0.27	96	7.65±0.32	96
150	7.94±0.26	7.71±0.60	97	7.68±0.29	96	7.62±0.26	96	7.59±0.15	95
180	7.95±0.31	7.68±0.38	96	7.64±0.16	96	7.56±0.21	95	7.50±0.28	94

Control, pH and bile salt values are mean $\log_{10}$ (cfu/ml) ± standard deviation of duplicate experiments

a = control cells in pH 7.3 (phosphate buffered saline)

b = SR (survival ratio), stressed cell number divided by control cell number multiplied by 100