CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND OF STUDY

Diarrhoea is a gastrointestinal tract (GIT) disorder which accounts for global deaths of nearly 1.5 million children under five years of age. It is the second most common cause of child death worldwide after pneumonia. Over half of the deaths are estimated to occur in five countries: India (386,600); Nigeria (194,000); Democratic Republic of Congo (89,900); Afghanistan (82,100); and Ethiopia (73,700) (Unicef, 2015).

The most common cause of diarrhoea is an infection of the intestines due to a bacterium, virus, parasite or fungus, a condition known as gastroenteritis (Ciccarelli *et al.,* 2013). These infections are often acquired from food or water that had been contaminated directly from another person who was infected with enteropathogens (WHO, 2013). Gastroenteritis is usually an acute and life threatening disease, resulting from loss of fluid and electrolytes particularly sodium ion (Na^+) and water from the body, which requires specific anti-infective therapy (Nwachukwu *et al.,* 2013). Enteric bacteria comprised of *Salmonella* species, *Shigella* species, *Proteus* species, *Klebsiella* species, *E. coli*, *Pseudomonas* species, *Vibrio cholerae* and *S. aureus* which are major aetiologic agents of enteric infections (Ballal and Shivananda, 2002).

Enteric infections are caused by two major bacterial mechanisms including mucosal invasion and secretion of toxins (Omojate *et al.,* 2014), are associated with Symptoms such as anorexia, nausea, vomiting, diarrhea, and abdominal discomfort and sometimes lactose intolerance (Lin *et al.,* 1998). Treatment and prevention of infectious gastroenteritis are not limited to antibiotics therapy since indiscriminate use fosters the emergence of drug-resistant organisms. Consequently, the European Society for Paediatric Gastroenterology, Hepatology and Nutrition and the European Society of

Paediatric Infectious Diseases guidelines made a strong recommendation for the use of probiotics for the management of acute gastroenteritis (Ciccarelli *et al.,* 2013).

Probiotics are microbial food supplements that prevent pathogens from proliferation in the intestinal tract on the surface structures, aid maximum feed utilization, improve water quality and enhance the immune system of the host (Nwogu *et al.,* 2011). Probiotics are adjuncts in health promoting food for humans and also used as therapeutic, prophylactic and growth supplements in animal production and human health (Isolauri, 2001; Amund, 2016).

More importantly, the emergence of antibiotic resistance in enteric pathogenic bacteria has facilitated the prophylactic and therapeutic uses of probiotics. Consequently, probiotics should be considered as alternatives to antibiotics.

1.2 STATEMENT OF THE PROBLEM

Enteropathogenic bacteria, which have been implicated in gastrointestinal diseases, are of global public health concern because of the significant morbidity and mortality especially in low and middle income countries, like Nigeria, where they place tremendous burdens on fragile healthcare systems. The major concern is the emergence of antibiotic resistant strains of enteric pathogens due to the widespread use of antibiotics. Consequently, alternative preventive and treatment measures for gastro intestinal disorders are required. Probiotics have been proven to be more effective in the management of acute gastroenteritis with no reported side effects. Probiotics are strain specific and not readily available in low income countries like Nigeria. Dependence on foreign probiotics is a challenge hence the need for isolation of indigenous strains for use.

1.3 AIM AND OBJECTIVES

The overall aim of the study is to isolate potential probiotic lactic acid bacteria from fermented sorghum (*Sorghum bicolor*) gruel and beverage.

1.4 SPECIFIC OBJECTIVES OF STUDY

The specific objectives of this research are to:

Isolate and identify putative beneficial bacteria from fermented sorghum product;

Examine the isolates for anti-enteropathogenic probiotic properties;

Characterize and genetically identify selected isolate(s);

Investigate and characterize the basis of antibacterial activity of identified isolate(s).

1.5 SIGNIFICANCE OF STUDY

Lactic acid fermentation is very important in food processing in the low and medium income countries, thus has the potential for use as a measure of disease control. In order to meet the increasing demand for viable food, lactic acid fermentation is expected to play an important role in preserving fresh vegetables, cereals, fruits and other food items for feeding humanity in developing countries of the world. Lactic acid bacteria (LAB) which have been reported as probiotic strains play a major role in fermentation, enhancing nutritive values of fermented products in addition to acidifying the products which prevents the growth of some spoilage and pathogenic organisms. However, probiotic properties are strain-specific. This study will provide indigenous LAB for local use as probiotics, alternative means of treatment and prevention of gastrointestinal disorders such as diarrhoea in low income countries including Nigeria. The prospective probiotic LAB isolates should be non-resstant to antibiotics since enteric LAB have been reported to demonstrate capability to transfer antibiotic resistance gene to unrelated enteric bacteria, which is factor contributing to pathogenesis. In addition, this research will reduce dependence on imported probiotics and antibiotics in the management and prevention of diarrhoea in Nigeria thereby saving cost. The study may, however, be a source of revenue from the export of this indigenous probiotics.

1.6 OPERATIONAL DEFINITION OF TERMS

Antibacterial: substance which inhibits growth of bacteria.

Antibiotics: commercially available chemical substance derived from a mold or bacterium that can inhibit growth of microorganisms.

Beverage: is a liquid food made from sorghum, boiled in water that may be more often drunk.

Bran: broken coat of seeds of sorghum.

Curing: to induce loss of plamid DNA.

Disorder: a physical condition in which there is imbalance of normal functioning.

Diarrhoea: is the condition of having at least three loose or liquid bowel movements each day which often lasts for a few days and can result in dehydration due to fluid loss.

Enteric: relating to the inside of the intestines.

Enzyme: a protein produced by a living organism that acts as catalyst in specific biochemical reactions.

Fermentation: is a metabolic process that converts sugar to acids, gases or alcohol

Filtrate: liquid substance that passes through a filter.

Gene: a segment of DNA that is involved in producing polypeptide chain.

Germ: a small simple structure from which a new tissue can develop into a whole organism.

Gruel: is a thinner version of food porridge made from sorghum.

Hull: a dry outer covering of sorghum seed.

Kiln: a heating mantle for drying grains

Mash: a mixture of bran and filtrate of sorghum.

Peptide: is a biologically occurring short chain of amino acids linked by peptide (amide) bonds.

Pomace: a crushed or ground, pulpy substance from wet-milled sorghum grains.

Probiotics: are microorganisms that provide health benefits when consumed by humans and animals.

Proteinaceous: possessing the physiochemical properties of proteins.

Surfactant: chemical substance capable of reducing surface tension of phospholipid bilayer component of microbial cell walls.

1.7 LIST OF ABBREVIATIONS

AFLP – Amplified Fragment Length Polymorphism

AIDS – Acquired Immune Deficiency Syndrome

AOAC – Association of Official Analytical Chemists

API – Analytical Profile Index

ARDRA - Amplified Ribosomal DNA Restriction Analysis

BLAST – Basic Local Alignment Search Tool

CDV – Cardiovascular Diseases

CoA – Coenzyme A

DNA – Deoxyribonucleic Acid

DP – Degree of Polymerisation

EcN – *Escherichia coli* Nissle

EDTA – Ethylene Diamine Tetraacetic Acid

EMP – Emden-Meyerhof Panas

ERIC – Enterobacterial Repetitive Intergenic Consensus

FAO - Food and Agriculture Organization of the United Nations

FOS – Fructo Oligosaccharides

GFN - Glucose, Fructose, number

GRAS - Generally Recognized As Safe

IBDs - Inflammatory Bowel Diseases

IPTG - Isopropyl β-D-1-thiogalactopyranoside

LAB – Lactic Acid Bacteria

LDC – Lowe Density Lipoprotein

MEGA – Molecular Evolutionary Genetics Analysis

MIC - Minimum inhibitory concentration

MRS – de Man Rogosa and Sharpe

MUB – Mucus-Binding Proteins

NAD – Nicotinamide Adenine Dinucleotide

NDOs – Non-Digestible Oligosaccharides

OB – *Ogi baba*

ORT – Oral Rehydration Therapy

PB – *Pito* Beverage

PCR – Polymerase Chain Reaction

PFGE – Pulse- Field Gel Electrophoresis

RAPD – Randomly Amplified Polymorphism DNA

rep-PCR – Repetitive Extragenic Palindromic PCR

RFLP – Restriction Fragment Length Polymorphism

RPM – Revolutions per minute

rRNA – ribosomal Ribonucleic Acid

SCFA – Short-Chain Fatty Acid

TAG - Triacylglycerol

UNICEF - United Nations Children's Fund

WHO – World Health Organization

X-GAL - 5 Bromo, 4 Chloro,3- Indolyl-D-Galactopyranoside

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 PROBIOTICS

The human gut flora consists of about 500 to 1,000 bacterial species (Sears, 2005; Sommer and Bäckhed, 2013), about 90% of which are obligate anaerobes (Kayser *et al.*, 2005). Probiotics have been suggested to protect against infectious diseases by several strain-dependent mechanisms (Foligne *et al.,* 2010) including secretion of antipathogen substances, competitive exclusion of pathogens, maintenance of mucosal integrity, and stimulation of systemic or mucosal immune responses (Howarth and Wang, 2013). The word probiotic is translated from the Greek meaning "for life". An early definition was given by Parker (1974): "Organisms and substances which contribute to intestinal microbial balance". However, this was subsequently refined by Fuller (1989) as: "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance". This latter version is the most widely used definition and has gained widespread scientific acceptability. Probiotics has a long history. In fact, the first records of intake of bacterial drinks by humans are over 2,000 years old. However, the idea of probiotics can be said to have emerged in the early 1900s as reported by Henry Tissier and Elie Metchnikoff (Oyetayo and Oyetayo, 2005). During these studies, he hypothesized that the normal GIT microflora could exert adverse effects on the host and that consumption of certain bacteria could reverse this effect (Anukam and Reid 2007). Metchnikoff refined the treatment by using pure cultures of what is now called *Lactobacillus delbruckeii* subspecie of *Lactobacillus bulgaricus*, which with *Streptococcus salivarius* subspecie of *Streptococcus thermophilus*, is used to ferment milk in the production of traditional yoghurt. Subsequent research has been directed towards the use of intestinal isolates of bacteria as probiotics (Fernandes *et al.*, 1987, Chandok *et al.,* 2015). Over the years many species of microorganisms have been used. They mainly consist

of lactic acid producing bacteria (lactobacilli, streptococci, enterococci, lactococci, bifidobacteria) (Afolabi *et al*., 2005), but also *Bacillus spp*. and fungi such as *Saccharomyces spp.* and *Aspergillus spp.* Despite the very widespread use of probiotics, the approach may have some difficulties. The bacteria used are usually anaerobic and do not tolerate extremes of temperature. To be effective, probiotic must be amenable to preparation in a viable form at a large scale. During use and under storage the probiotic should remain viable and stable, and be able to survive in the intestinal ecosystem. In addition, the host animal should gain beneficially from harbouring the probiotic. It is therefore proposed that the exogenous bacteria reach the intestine in an intact and viable form in order to exert their advantageous properties (Fooks and Gibson, 2002). To exert probiotic properties, microbes must overcome a number of physical and chemical barriers in the GIT, which include gastric acidity and bile acid secretion. Moreover, on reaching the colon the probiotics may be in some sort of stressed state that would probably compromise chances of survival (Al-Salami *et al.,* 2012).

2.1.1 PROBIOTIC MICROORGANISMS WITH BENEFICIAL PROPERTIES

Consequent to several allusions the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) defined probiotics as live microorganisms, which when administered in adequate amounts, confer a health benefit on the host (Joint FAO/WHO, 2002). This definition of probiotics is also adopted by the International Scientific Association for Probiotics and Prebiotics (ISAPP) and is used in most scientific publications.

Although the benefits of consuming fermented foods have been known to mankind for centuries; long before microorganisms were discovered; the concept of administering microorganisms in order to confer a positive health benefit started over a century ago when Metchnikoff theorized that health could be enhanced, and also senility/aging could be delayed, by manipulating the

intestinal microflora with host-friendly bacteria found in fermented foods, like yogurt (Mackowiak, 2013).

The most common types of microorganisms used as probiotics are lactic acid bacteria, although other bacteria and certain yeasts are also used (Isolauri *et al.,* 2004; Didari, 2014; Emerenini *et al.,* 2014). It is important to stress that the biological effects of probiotics are strain specific and that the success or failure of one strain cannot be extrapolated to another strain (Agrawal, 2005).

Additionally, a number of health benefits have been claimed for lactic acid bacteria and therefore inclusion of these organisms in the diet is considered to be important in maintaining good health (Champagne *et al.*, 1996). Probiotics have anticarcinogenic properties, a specific probiotic effect, which are of three types:

(1) elimination of procarcinogens;

(2) modulation of procarcinogenic enzymes; and

(3) tumour suppression (Wollowski *et al.*, 2001; Enujiugha and Badejo, 2017).

2.1.2 PROBIOTIC PROPERTIES OF MICROORGANISMS

2.1.2.1 Genus *Lactobacillus*

The genus *Lactobacillus* includes various Gram positive facultative anaerobic or microaerophilic rod-shaped bacteria. They are a major part of the lactic acid bacteria (LAB) group (including *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Oenococcus*, *Pediococcus*, *Streptococcus* and *Leuconostoc* species) that can convert hexose sugars to lactic acid thus producing an acidic environment which inhibits the growth of several species of harmful bacteria (Makarova, 2006).

Lactobacilli are non-sporulating bacteria that show remarkable ecological adaptability and phylogenetic diversity (Mayo *et al*., 2014). They are commonly found in human body cavities,

such as vagina, intestinal tract and oral cavity. They also naturally persist in a broad range of food environments, such as fermented milk, meat and plant (Oranusi *et al*., 2014). A number of lactobacilli species have been extensively implemented in various industrial processes, as starter or adjunct cultures (Stiles and Holzapfel, 1997).

Recently, some strains of the species *Lactobacillus casei*, *L. paracasei*, *L. reuteri*, *L. rhamnosus*, *L. acidophilus, L. plantarum* and *L. johnsonii* have also been increasingly employed in food products marketed as probiotics (Chukwu *et al.*, 2014). The health benefits associated with the consumption of products containing some of these commercialized strains of lactobacillus have been demonstrated by a number of human clinical studies on patients with diverse disorders (Enujiugha and Badejo, 2017). However, the large diversity of lactobacilli also suggested that probiotic functions and health-promoting properties are specific to each strain, justifying the need to examine probiotic lactobacilli strains individually on a genomic and molecular basis (Ventura *et al*., 2009).

Some lactobacilli are used for the production of yogurt, cheese, sauerkraut, pickles, sourdough, wine and other fermented products (Mayo *et al*., 2008). In all cases, sugars are metabolized into lactic acid; thus creating a hostile environment for spoilage microorganisms and enabling food preservation (De Vuyst *et al*., 2014; Iroanya, 2015).

Lactobacilli have received tremendous attention due to their health-promoting properties. However, a very important fact that is sometimes overlooked by scientists is that most lactobacilli do not form stable and numerically significant populations in the human intestinal tract, especially in the small intestine where they are presumed to form epithelial associations (Walter, 2008).

Lactobacilli such as: *Lactobacillus acidophilus*, *L. casei*, *L. paracasei*, *L. rhamnosus*, *L. delbrueckii* subsp. *bulgaricus*, *L. brevis*, *L. johnsonii*, *L. plantarum* and *L. fermentum* are commonly used as probiotic products. Although lactobacilli are often described as indigenous inhabitants of the human intestinal tract, they are more likely to be autochthonous of the oral cavity or fermented foods (Fijan, 2014).

Studies have shown that certain strains of lactobacillus are effective in preventing antibiotic-associated diarrhoea (Hempel *et al.,* 2012; Butel, 2014). *Lactobacillus* species are commonly selected as probiotics since they express many crucial properties such as: high tolerance to acid and bile, capability to adhere to intestinal surfaces, withstanding low pH, gastric juice, inhibiting potentially pathogenic species (antimicrobial activity), resisting antibiotics, producing exopolysaccharides and removing cholesterol (Banwo and Tan, 2013; Tulumoglu *et al.,* 2013).

Sometimes probiotic 'cocktails' comprising of various strains are used (McFarland*,* 2007; Relman, 2013). Several probiotic strains of microorganisms are effective in competing against common causes of travellers' diarrhoea caused mostly by bacterial pathogens such as: one of the seven types of diarrhoeagenic *Escherichia coli*, *Campylobacter jejuni* and *Shigella* species (McFarland, 2007; Amenu, 2015).

2.1.2.2 Genus *Bifidobacterium*

The genus *Bifidobacterium* includes various Gram positive non-motile anaerobic bacteria. They are endosymbiotic inhabitants of the gastrointestinal tract and vagina of mammals, including humans (Chen *et al.,* 2007; Fijan, 2014). Strains of the genus *Bifidobacterium* are also often used as probiotic bacteria as they are known for their variety of resistance mechanisms to bile salts, which is important since the beneficial effects of probiotic bacteria must be generated in the presence of this biological fluid (Fijan, 2014).

It has even been proven that although bile tolerance is strain dependent, both wild type-bile sensitive bifidobacteria and lactobacilli strains can progressively adapt to the presence of bile salts by subculturing and gradually increasing concentration of bile (Noriega *et al.*, 2006; Ruiz *et al.,* 2013). Several strains of bifidobacteria are considered as important probiotics including: *Bifidobacterium infantis*, *B. adolescentis*, *B. animalis* subsp *animalis*, *B. animalis* subsp *lactis*, *B. bifidum*, *B. longum*, *B. breve* (Mayo *et al.,* 2014).

Bifidobacterium species together with other probiotics have been proven to treat constipation (Chmielewska and Szajewska, 2010; Enujiugha and Badejo, 2017), travelers' diarrhoea (McFarland, 2007; Mayo and Alonso 2015), antibiotic-associated diarrhoea (Hempel *et al.*, 2012; Jafarnejad *et al.,* 2016), maintaining remission of disease activity of gut inflammation and moderate ulcerative colitis (Aloisio *et al.*, 2012; Dylag *et al.*, 2014), prevention as well as treatment of necrotizing enterocolitis in newborns (Di Gioia *et al.*, 2014), reduction of radiation induced diarrhoea (Demers *et al.*, 2013), reducing the development of disease risk for eczema, food allergies (Isolauri *et al.,* 2012), cholesterol-lowering capacities (Ruiz *et al.*, 2013).

2.1.2.3 Genera *Streptococcus* and *Enterococcus*

The genera *Streptococcus* and *Enterococcus* are also part of the lactic acid bacteria and contain several strains associated with severe health-care associated infections such as: *Streptococcus pyogenes*, *Streptococcus pneumoniae*, vancomycin-resistant *Enterococcus faecium* (Hossain, 2014). However, other strains form part of the commensal human microbiome of the mouth, skin, and intestine, such as *Enterococcus faecium* PC4.1 (Hadji-Sfaxi *et al.*, 2011) and others. Some strains have probiotic properties such as *Enterococcus durans* and *Streptococcus thermophilus* (Pieniz *et al.*, 2013) (also used for the production of yogurt alongside *Lactobacillus delbrueckii* subsp.*bulgaricus*).

Although *Enterococcus faecium* has a long history of probiotic use, especially in preventing antibiotic-associated diarrhoea (Hempel *et al.*, 2012), certain strains are opportunistic pathogens that present a potential pool of antibiotic resistance and virulence genes, in animals (Dirienzo, 2014) and are therefore not generally recognized as safe (GRAS) for humans, but represent important probiotics for animals (Bednorz *et al.*, 2013; Cao *et al.*, 2013).

2.1.2.4 Genus *Lactococcus*

Genus *Lactococcus* are a group of Gram positive lactic acid bacteria that are commonly used in the dairy industry for manufacturing fermented products. They are important in preventing growth of spoilage bacteria in milk products due to acidification. They are also sometimes recommended as probiotics. Certain strains of *Lactococcus lactis* subsp. *lactis* have probiotic properties such as adhesion to vaginal epithelial cells and nisin production (*Lactococcus lactis* subsp. *lactis* CV56) (Gao *et al.*, 2011; Yang *et al.*, 2013) and are also used to treat antibiotic-associated diarrhoea in combination with other probiotics (Johnston *et al.*, 2011).

2.1.2.5 Genus *Bacillus*

The genus *Bacillus* includes Gram positive spore-forming aerobic or facultative aerobic members with probiotic properties including: *B. subtilis*, *B. coagulans*, *B. subtilis*, *B. cereus*. *Bacillus coagulans* together with other microorganisms has proven to be most successful in preventing or treating antibiotic-associated diarrhoea (Hempel *et al*., 2012).

Bacillus subtilis spores have been considered as probiotics for animal consumption (Larsen *et al.*, 2013; Zokaeifar *et al.*, 2014) and have been proposed for treating diarrhoea and *H. pylori* eradication in humans (Tompkins *et al.*, 2010).

2.1.2.6 Genus *Escherichia*

Although the genus *Escherichia,* which belongs to the Gram negative family Enterobacteriaceae, is mainly known for its severely virulent serotypes (e.g., *E. coli* O157:H7), *Escherichia coli* is a very common inhabitant of the lower intestine. A particular probiotic strain has been identified as *Escherichia coli* Nissle 1917 (EcN) which when administered alongside other probiotics have been proven to treat constipation (Chmielewska and Szajewska, 2010) and inflammatory bowel disease (Behnsen *et al.*, 2013). This strain has also been found to relieve gastrointestinal disorder, ulcerative colitis, Crohn's disease (Xia *et al*., 2013), even colon cancer (Behnsen *et al.*, 2013), however more research is necessary.

2.1.2.7 Genus *Saccharomyces*

The genus *Saccharomyces* includes various yeasts such as: *Saccharomyces cerevisiae* (used for making wine, bread, beer) (Gibson, 2010), *Saccharomyces bayanus* (used for making wine) (Nguyen *et al.*, 2011) and *Saccharomyces boulardii* used in medicine as a probiotic (Dinleyici *et al.*, 2012). *Saccharomyces* yeasts also form symbiotic matrices with bacteria to form non-alcoholic fermented foods like *kefir* (Witthuhn *et al.*, 2005; de Oliveira *et al.*, 2013) and are sometimes a component of *kombucha* (Marsh *et al.*, 2014).

S. boulardii is often marketed as a probiotic in a lyophilized form to treat diarrhoea while maintaining an excellent reputation for safety (McFarland, 2007; De Oliveira *et al.*, 2013). Most reports show a clinical benefit of *S. boulardii* in decreasing the duration of diarrhoea regardless of the cause and thus reducing hospital stay resulting in social and economic benefits (Szajewska *et al.*, 2005; Johnston *et al.*, 2011; Vieira *et al.*, 2012; Shan *et al.*, 2013). Administration of *S. boulardii* has shown positive effects for patients with irritable bowel syndrome (Choi *et al.,* 2011), preventing and treating relapses of inflammatory bowel disease and for treating moderate

symptoms of ulcerative colitis (Guslandi *et al.*, 2003). Recurrent pseudomembranous colitis infection caused by *Clostridium difficile* can also be significantly reduced by administration of daily dosages of *S. boulardii* together with standard antibiotics (Fitzpatrick, 2013).

The most common probiotic microorganisms with claimed beneficial properties for humans from the most scientific literature are presented in Table 1 (Fijan, 2014).

Table 1: Health benefits of probiotic microorganisms

Genus	Species	Health Benefits
Lactobacillus	*L. rhamnosus*	Reduction of viral-associated pulmonary damage (*L. rhamnosus* CRL1505); prevention and reduction of severity of atopic dermatitis in children (*L. rhamnosus* GG); reduction of risk for developing allergic disease (*L. rhamnosus* GG), (*L. rhamnosus* HN001; anti-diabetic potential (various strains from human infant faecal samples); prevention of necrotizing enterocolitis in newborns (*L. rhamnosus* GG); prevention or treatment of bacterial vaginosis (*L. rhamnosus* GR-1); aid in weight loss of obese women (*L. rhamnosus* CGMCC1.3724); treatment of acute gastroenteritis in children (*L. rhamnosus* GG); reduction of risk for rhinovirus infections in preterm infants (*L. rhamnosus* GG and *L. rhamnosus* ATCC 53103); protection of human colonic muscle from lipopolysaccharide-induced damage (*L. rhamnosus* GG).
	L. acidophilus	Treatment of travellers' diarrhoea; reduction of hospital stay of children with acute diarrhoea;

Genus	Species	Health Benefits
		antifungal activity (*L. acidophilus* ATCC-4495); prevention or treatment of bacterial vaginosis; treatment of *C. difficile*-associated diarrhoea; reduction of incidence of febrile urinary tract infections in children; reduction of irritable bowel syndrome symptoms.
	L. plantarum	Prevention of endotoxin production; antifungal activity *(L. plantarum* NRRL B-4496) reduction of irritable bowel syndrome symptoms.
	L. casei	Treatment of functional constipation in adults (*L. casei* Lcr35 and *L. casei* Shirota); treatment of *C. difficile*-associated diarrhoea; restoration of vaginal flora of patient with bacterial vaginosis (*L. casei* Lcr35); reduction of irritable bowel syndrome symptoms; reduction of diarrhoea duration of antibiotic-associated diarrhoea in geriatric patients (*L. casei* Shirota); immunomodulatory mechanisms (*L. casei* Shirota); improvement of rheumatoid arthritis status (*L. casei* 01); protection against *Salmonella* infection (*L. casei* CRL-431); prevention of *Salmonella*-induced synovitis; treatment of intravaginal staphylococcosis (*L. casei* IMV B-7280).
	L. delbrueckii subsp. *Bulgaricus*	Antibiotic resistance of yogurt starter culture; enhancement of systemic immunity in elderly (*L. delbrueckii* subsp. *bulgaricus* 8481);

Genus	Species	Health Benefits
		antibacterial action against *E. coli*; modulation of brain activity.
	L. brevis	Protective role in bile salt tolerance (*L. brevis* KB290); reduction in plague acidogenicity (*L. brevis* CD2).
	L. johnsonii	Impact on adaptive immunity for protection against respiratory insults; reduction of occurrence of gastritis and risk of *H. pylori* infection (*L. johnsonii* MH-68); inhibition of *S. sonnei* activity (*L. johnsonii* F0421); treatment of perennial allergic rhinitis in children together with levocetirizine (*L. johnsonii* EM1).
	L. fermentum	Prevention or treatment of bacterial vaginosis (*L. fermentum* RC-14); blockage of adherence of pathogenic microorganisms on vaginal epithelium; antistaphylococcal action (*L. fermentum* ATCC 11739); potential for reduction of insulin resistance and hypercholesterolemia (*L. fermentum* NCIMB 5221).
	L. reuteri	Reduction of low-density lipoprotein cholesterol (*L. reuteri* NCIMB 30242); treatment of acute gastroenteritis in children; reduction of diarrhoea duration in children (*L. reuteri* ATCC 55730); management of infant colic (*L. reuteri* ATCC 55730 and *L. reuteri* DSM 17938); reduction of onset of gastrointestinal disorders in infants (*L. reuteri* DSM 17938); reduction of frequency of

Genus	Species	Health Benefits
		proven sepsis, feeding intolerance and duration of hospital stay in preterm infants (*L. reuteri* DSM 17938).
Bifidobacterium	*B. infantis*	Reduction of irritable bowel syndrome symptoms; reduction of necrotizing enterocolitis in preterm infants.
	B. animalis subsp. *Lactis*	Treatment of functional constipation in adults (*B. animalis* subsp. *lactis* DN-173 010); reduction of incidence of febrile urinary tract infections in children; modulation of brain activity; reduction of necrotizing enterocolitis in preterm infants; reduction of total microbial counts in dental plaque (*B. animalis* subsp. *lactis* DN-173 010); reduction of total cholesterol (*B. animalis* subsp. *lactis* MB 202/DSMZ 23733) ; reduction of risk of upper respiratory illness (*B. animalis* subsp. *lactis* BI-04).
	B. bifidum	Reduction of hospital stay of children with acute diarrhoea; reduction of necrotizing enterocolitis in preterm infants; reduction of total cholesterol (*B. bifidum* MB 109/DSMZ 23731).
	B. longum	Prevention and treatment of necrotizing enterocolitis in newborns; reduction of radiation induced diarrhoea; reduction of necrotizing enterocolitis with Bifidobacteria cocktail (*B. breve*, *B. infantis*, *B. bifidum*, *B. longum*); reduction of irritable bowel syndrome

Genus	Species	Health Benefits
		symptoms; treatment of gastrointestinal diseases (*B. longum* CMCC P0001); perinatal intervention against onset of allergic sensitization (*B. longum* CCM 7952).
	B. breve	Prevention and treatment of necrotizing enterocolitis in newborns; reduction of necrotizing enterocolitis with Bifidobacteria cocktail (*B. breve*, *B. infantis*, *B. bifidum*, *B. longum*); reduction of cholesterol (*B. breve* MB 113/DSMZ 23732).
Saccharomyces	*S. boulardi*	Treatment of travellers' diarrhoea; treatment and reduction of diarrhoea duration regardless of cause; treatment of irritable bowel syndrome; treatment of moderate ulcerative colitis; treatment and reduction of recurrent pseudomembrane colitis infection caused by *C. difficile*; treatment of acute gastroenteritis in children.
Lactococcus	*L. lactis* subsp. *Lactis*	Treatment of antibiotic-associated diarrhoea; adhesion to vaginal epithelial cells (*L. lactis* subsp. *lactis* KLDS4.0325); nisin production (*L. lactis* subsp. *lactis* CV56); modulation of brain activity; antimicrobial activity against *C. difficile*; antimicrobial and probiotic properties (*L. lactis* subsp. *lactis* ATCC 11454).

Genus	Species	Health Benefits
Enterococcus	*E. durans*	Antibiotic and antioxidant activity (*E. durans* LAB18s), adherence to colonic tissue and anti-inflammatory activity.
	E. faecium	Treatment of antibiotic-associated diarrhoea; efficient animal probiotic.
Streptococcus	*S. thermophiles*	Reduction of irritable bowel syndrome symptoms; antibiotic resistance of yogurt starter culture; reduction of necrotizing enterocolitis in preterm infants.
Pediococcus	*P. acidilactici*	Pediocin production with antimicrobial and probiotic properties (*P. acidilactici* UL5); bacteriocin production; elimination of *H. pylori* infections (*P. acidilactici* BA28).
Leuconostoc	*L. mesenteroides*	Leucoin production, probiotic profile (survival at low pH, in presence of bile salts, in presence of pepsin) (*L. mesenteroides* B7).
Bacillus	*B. coagulans*	Treatment of antibiotic-associated diarrhoea, treatment of bacterial vaginosis (*B. coagulans* ATCC PTA-11748); immunological support (*B. coagulans* GandenBC30); prevention of caries in children.
	B. subtilis	Efficient animal probiotic; treatment of diarrhoea and aiding in *H. pylori* eradication (*B. subtilis* R0179); production of nitric oxide.
	B. cereus	Efficient animal probiotic (*B. cereus* NVH75/95).

Genus	Species	Health Benefits
Escherichia	*E. coli* Nissle 1917	Treatment of functional constipation in adults; treatment of inflammatory bowel disease; treatment of gastrointestinal disorders; pro-inflammatory potential; prevention of surface ocular diseases; reduction of *Salmonella enterica* Typhimurium intestinal colonization by iron competition.

2.1.3 REQUIREMENTS FOR PROBIOTICS

It is of great significance that the probiotic strain can survive the location where it is presumed to be active. For a longer and perhaps higher activity, it is necessary that the strain can proliferate and colonise at this specific location. Probably only host-specific microbial strains are able to compete with the indigenous microflora and to colonise the niches (Eric and Byong, 2015).

The properties expected from potential probiotic strains of lactic acid bacteria, compiled by many authors (Havenaar *et al*., 1992; Veld and Shortt, 1996; Joint FAO/WHO, 2002; Sarkar, 2010; Seale and Millar, 2013; Tulumoglu *et al.*, 2013; Anwar *et al*., 2014) are:

a) Accurate taxonomic identification;

b) Normal inhabitant of the species targeted human origin for human probiotics;

c) Nontoxic and non-pathogenic;

d) Genetically stable;

e) Capable of survival, proliferation, and metabolic activity at the target area;

f) Adherence and colonization potential preferred;

g) Stability of desired characteristics during culture preparation, storage, and delocalization;

h) Viability at high populations;

i) Production of antimicrobial substances, as well as bacteriocins, hydrogen peroxide and organic acids;

j) Antagonistic for pathogenic/cariogenic bacteria;

k) Able to compete with the normal microflora, as well as the same or closely related species; potentially resistant to acid, bacteriocins and other antimicrobials produced by residing microflora;

l) Resistant to bile;

m) Resistant to acid;

n) Immunostimulatory;

o) Able to exert one or more clinically documented health benefits;

p) Amenable to production processing adequate growth, concentration, recovery, freezing, dehydration, storage and distribution;

q) Provision of suitable organoleptic qualities when included in fermented products.

2.1.4 VIABILITY OF PROBIOTIC ORGANISMS

Microorganisms ingested have to transiently survive in the stomach and small intestine. Although this appears to be a rather minimal requirement, many bacteria including the yoghurt-producing bacteria *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* often do not survive to reach the lower small intestine (Elli *et al.*, 2006). The reason for this appears to be low pH of the stomach. In fasting individuals, the pH of the stomach is between 1.0 and 2.0 and most microorganisms, including lactobacilli, can only survive from 30 seconds to several minutes under these conditions (Desalegn, 2014). Therefore, in order for a probiotic to be effective, even the selection of strains that can survive in acid at pH 3.0 for some time would have to be introduced in a buffered foods such as milk, yoghurt or fermented products (Desalegn, 2014).

2.1.4.1 Antimicrobial Properties

The intestinal microflora is a complex ecosystem, and introducing new organisms into this highly competitive environment is difficult. Thus, organisms that can produce a product that inhibits the growth of existing organisms have a characteristic advantage. The ability of probiotics to establish in the GIT is enhanced by their ability to eliminate competitors. In different studies on humans and animals, beneficial microorganisms are used to improve the colonization resistance on body surfaces, such as GIT, the urogenital, and the respiratory tract. Bifidobacteria produce acetic and lactic acids in a molar ratio of 3:2 (Desjardins and Roy, 1990). *Lactobacillus acidophilus* and *Lactobacillus casei* produce lactic acid as the main end product of fermentation. In addition to lactic and acetic acids, probiotic organisms produce other acids, such as hippuric and citric acid. Lactic acid bacteria also produce hydrogen peroxide, diacetyl, and bacteriocin as antimicrobial substances. These inhibitory substances create antagonistic environments for foodborne pathogens and spoilage organisms. Yoghurt bacteria are reported to produce bacteriocin against probiotic bacteria and vice versa (Dave and Shah, 1997).

2.1.4.2 Acid and Bile Tolerance

One of the most important criteria for selection of probiotic organisms is their ability to survive in the acidic environment of the product and in the stomach, where the pH can reach as low as 2. Similarly, the organisms must be able to survive in the bile concentrations encountered in the intestine. Studies have shown that a few strains of lactic acid bacteria survived under the acidic conditions and bile concentrations normally encountered in fermented products and in the GIT (Clark *et al.*, 1993; Lankaputhra and Shah, 1995). Therefore, it cannot be generalized that all probiotic strains are acid and bile tolerant.

2.1.4.3 Anticarcinogenic Properties

In the last two decades, the number of people suffering from colon cancer has been gradually increasing, particularly in developing countries (Torre *et al.,* 2015). Studies by Goldin and Gorbach (1984) have indicated that diet and antibiotics can lower the generation of carcinogens in the colon and reduce chemically induced tumours (Goldin and Gorbach, 1981; Goldin and Gorbach, 1984). These effects appear to be mediated through the intestinal microflora. Additional studies have shown that the introduction of *L. acidophilus* into the diet lowered the incidence of chemically induced colon tumours in rats (Goldin and Gorbach, 1980). A possible mechanism for these anticancer effects relies on inhibiting intestinal bacterial enzymes that convert procarcinogens to more proximal carcinogens (Kumar *et al.*, 2011). This technique can be expanded in the future by testing probiotics for their ability to inhibit the growth or organisms normally found in the flora that have high activities of enzymes such as β-glucuronidase (Reddy *et al*., 1974; Reddy, 1999), nitroreductase, azoreductase and β-glycosidase or the capability for nitrososation (Reddy, 1999).

The sixth most commonly diagnosed cancer in the world is hepatitis B virus. Consumption of foods, contaminated with aflatoxins, is also established causes of liver cancer. Aflatoxin B1 (AFB1) causes characteristic genetic changes in the p53 tumor suppressor gene and ras protooncogenes. Some probiotic bacterial strains have been successfully shown to bind and neutralize AFB1 in vivo and thus reduce the bioabsorption of the toxin from the gut (Kumar *et al.*, 2011).

2.1.4.4 Adherence of Probiotic Bacteria

The ability of microorganisms to adhere to epithelial cells is to a large extent species specific, although this may be relative. The selection of human bacterial isolates will enhance the possibility

of finding organisms that will survive. There are several tests for determining if a prospective probiotic can bind to intestinal epithelium (Desai, 2008). Radiolabelling the microorganisms with an amino acid and then counting for adhering radioactivity in either ileal cells recovered from ileostroma effluent or from buccal cells obtained by gently scraping the inside of the cheek are effective methods (Desalegn, 2014). Good adhesion properties should enhance the possibility of long-term survival of the organism in the intestinal tract by countering the peristaltic action of the intestine (Nagpal *et al.*, 2012).

2.1.4.5 Immunological Enhancement

In recent years there have been several reports indicating that lactobacilli used in dairy products can enhance the immune response of the host. Organisms that have been identified as having this property are *Bifidobacterium longum, L. acidophilus, L. casei subsp. rhamnosum* and *L. helveticus* (Isolauri *et al*., 2001).

Enhanced phagocytic activity of granulocytes, cytokine excretion in lymphocytes, and increased immunoglobulin-secreting cells in blood are typical responses to probiotics, all of which are indicative of changes in the immune system (Nagpal *et al.*, 2012). An inflammatory immune response produces cytokine-activated monocytes and macrophages, causing the release of cytotoxic molecules capable of lysing tumor cells *in vitro* (Philip and Epstein, 1986). The inflammatory cytokines IL-1 and TNF-a exert cytotoxic and cytostatic effects on neoplastic cells in *in vitro* models (Raitano and Kore, 1993). Because immune function declines with age, enhancing immunity in the elderly with probiotics would be of particular use (Gill and Rutherfurd, 2001). Regardless of the mechanisms involved, probiotics cultures have been shown to stimulate both nonspecific immunity and specific immunity. Possible stimulation of an immune response by

probiotic bacteria may explain potential therapeutic and prophylactic applications of such cultures in the treatment for infections and carcinogenesis (Nagpal *et al.*, 2012).

2.1.4.6 Cholesterol Lowering

Coronary heart diseases and cardiovascular diseases (CVD), major causes of most death in adults, are conditions in which the main coronary arteries supplying the heart are no longer able to supply sufficient blood and oxygen to the heart muscle (myocardium) (Nagpal *et al.*, 2012). Although low-fat diets offer an effective means of reducing blood cholesterol concentrations, these appear to be less effective, largely due to poor compliance, attributed to low palatability and acceptability of these diets by the consumers (Nagpal *et al.*, 2012). Therefore attempts have been made to identify other dietary components that can reduce blood cholesterol levels. Individuals with CVD and those with a higher risk of developing the condition are treated in a number of ways to help lower their LDL cholesterol and triacylglycerol (TAG) concentrations while elevating their high-density lipoprotein cholesterol (Lin *et al.*, 2013).

However, since 1974 when Mann and Spoerry showed an 18% fall in plasma cholesterol levels after feeding 4–5 liters of fermented milk per day for 3 weeks to Maasai warriors, there has been a considerable interest in the effect of probiotics on human lipid metabolism (Mann and Spoerry, 1974). Supplementation of diet with dairy products fermented with LAB has the potential to reduce serum cholesterol levels in humans and animals (Pulusoni and Rao, 1983). The probable mechanisms of action of probiotics, in this regards, are cholesterol assimilation by bacteria, deconjugation of bile salts, cholesterol binding to bacterial cell walls, and reduction in cholesterol biosynthesis (Pulusoni and Rao, 1983; Pereira and Gibson, 2002).

2.1.4.7 Enhancement of Short-Chain Fatty Acid Production

Because the improved intestinal microbial communities with probiotics primarily involve the stimulation of intestinal fermentation, the stimulation of short-chain fatty acid (SCFA) production is one of the essential factors for the beneficial effects exerted by probiotics (Nagpal *et al.*, 2012). A significant increase in indigenous lactobacilli in the large intestine, as a result of probiotic Lactobacillus, causes increase in lactobacilli which stimulate lactate production (Tsukahara and Ushida, 2001). Although lactate does not accumulate in the large intestine, except in those patients with short bowel syndrome and dyspeptic diarrhoea (Tsukahara and Ushida, 2001). Rather, lactate is normally metabolized to acetate, propionate, or butyrate by lactate-utilizing bacteria (Bourriaud *et al.*, 2005; Belenguer *et al.*, 2006).

Lactate-utilizing bacteria from the human flora have been previously identified as belonging to the Clostridia cluster XIVa, based on their 16S rRNA gene sequences (Duncan *et al*., 2004). The increase in fecal SCFA by probiotic Lactobacillus would be due to this mechanism (Tsukahara *et al*., 2006). In fact, the oral administration of the lactate-utilizing and butyrate-producing bacterium, *Megasphaera elsdenii*, with *Lactobacillus plantarum* has been shown to increase the butyrate production in the large intestine. Thus, the administration of probiotics with other lactate-utilizing bacteria, butyrate-producing bacteria, in particular, could be a more effective way to achieve maximum health benefits (Nagpal *et al.*, 2012).

2.1.4.8 Production of Hormones and Other Agents

The possibility of genetically engineering strains of bacteria that can produce substances such as insulin, androgens, estrogens, growth hormone or cholesterol-lowering compounds, just to mention a few, is intriguing. The ability to produce drugs or hormones *in situ* over a long period of time by individuals suffering from various diseases (i.e. diabetes and hypercholesteremia) is of

particular interest (Desai, 2008). These are problems to this approach, however; e.g. control of production and contamination of normal individuals with the organism. The contamination problem may be more difficult to solve, although antibiotic sensitivity can be introduced into the strains, so that the organism could be rapidly eliminated if a normal individual is infected with a specifically designed probiotic (Desia, 2008). This idea may have too many regulatory problems associated with it; however, it is still something that may have potential use in human disease regulation.

2.1.4.9 Colonization Resistance

The indigenous microflora on body surfaces inhibit the colonization of non-indigenous microorganisms (Berg and Savage, 1972; Havenaar and Huis, 1992). Nevertheless, in some cases (potential) pathogenic microorganisms are able to penetrate and/or colonise these body surfaces, due to a massive attack of the pathogens or to a (temporarily) reduced colonization resistance (Havenaar and Huis, 1992; Letarov and Kulikov, 2009). In different studies on humans and animals beneficial microorganisms are used to improve the colonisation resistance on body surfaces, such as gastrointestinal-, the urogenital-, and the respiratory-tract (Havenaar and Huis, 1992; Nagpa *et al.*, 2012).

2.1.4.10 Diabetes and Obesity Management

The role of gut flora in the pathology of insulin resistance (type 2 diabetes) and obesity has been well documented by Ley *et al*. (2005). Animal and human studies have suggested that gut flora enhances the body weight gain and increases the insulin resistance, and these phenotypes are transmittable with gut flora during the implantation studies of microbiota from obese to normal and germ-free mice (Ley *et al.*, 2006; Turnbaugh *et al.*, 2006). The mechanisms associated with gut flora–mediated pathology of obesity and diabetes are through: increased energy harvest;

increased blood LPS levels (endotoxemia) and low-grade inflammation (Delzenne *et al.*, 2011). Therefore, modulation of gut flora has been considered as a potential target to treat against obesity and diabetes. Probiotics are novel gut flora modulators, and their role in the prevention of and treatment for diabetes and obesity has been implicated in recent past by Yadav *et al*. (2008). Probiotic-supplemented fermented milk product called *dahi* (yogurt) dramatically suppressed diet-induced insulin resistance and protected from streptozotocin induced diabetes in animal models in addition to enhancement of antioxidant system (Yadav *et al.*, 2008).

Studies have suggested that probiotic-based selective strains of Lactobacilli and Bifidobacteria show beneficial effects on obesity and type-2 diabetes (Aronsson *et al.*, 2010). Andreasen *et al.* (2010) reported that L. acidophilus decreased the insulin resistance and inflammatory markers in human subjects. More recently, Vajro *et al.* (2011) and others (Kang *et al.*, 2010; Chen *et al.*, 2011) showed that feeding of specific strains of Lactobacilli and Bifidobacteria ameliorate the progression of obesity and diabetes, suggesting that probiotic-mediated modulation of gut flora can be a potential therapy against obesity and diabetes. Also, the mechanism(s) of action for probiotic-based formulation is not completely understood; therefore, future studies should also be focused on describing the probiotic action–targeted molecules and organs in physiologic models.

2.1.4.11 Lactose Intolerance Management and other Potential Benefits

Certain functional foods containing probiotic provide preformed lactase to gut and allow better digestion of lactose (Nagpal *et al.*, 2012). The regulatory role of probiotics in allergic disease was demonstrated by a suppressive effect on lymphocytes' proliferation and interleukin-4 generation in vitro (Sutas *et al.*, 1996). Subsequently, the immune inflammatory responses to dietary antigens in allergic individuals were shown to be alleviated by probiotics, this being partly attributable to enhance the production of antiinflammatory cytokines (Pessi *et al*., 2000) and transferring growth

factor-B (Haller *et al*., 2000). Probiotic bacteria also possess prophylactic and therapeutic properties. Other potential benefits include protection against vaginal or urinary tract infections, reduction in ulcers and intestinal tract infections, increased nutritional value, maintenance of mucosal integrity, reduction in catabolic products eliminated by kidney and liver, stimulation of repair mechanism of cells, breaking down and rebuilding hormones, relieving anxiety and depression, formation, maintenance, or reconstruction of a well-balanced indigenous intestinal and/or respiratory microbial communities, inhibiting decalcification of the bones in elderly people, and synthesis of vitamins and predigestion of proteins (Sharma and Devi, 2014).

2.2 PREBIOTICS

The term `prebiotic' was first coined by Gibson and Roberfroid, hence defined as a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon that can improve the host health (Gibson and Roberfroid, 1995). The function of prebiotics is to basically stimulate existing metabolisms in the colon (Coussement, 1996).

The current interest in improving the host health by managing the colonic microflora calls for an alternative approach, which leads to consumption of food ingredients known as prebiotics (Rycroft *et al.*, 2001). Prebiotics, as currently considered and are all carbohydrates of relatively short chain length (Cummings *et al.*, 2001). Additionally, carbohydrates that have escaped digestion in the upper gastrointestinal tract form the predominant substrates for bacterial growth in the colon (Roberfroid *et al.*, 1998). Present evidence concerning the two most studied prebiotics, fructo-oligosaccharides and inulin, is consistent with their resisting digestion by gastric juice and pancreatic enzymes *in vivo*. In the large intestine prebiotics, in addition to their selective effects on LAB, influence many aspects of bowel function through fermentation (Campbell *et al.*, 1997;

Alles, 1998). Short-chain fatty acids are a major product of prebiotic breakdown, but as yet, no characteristic pattern of fermentation has been identified. Through stimulation of bacterial growth and fermentation, prebiotics affect bowel habit and are mildly laxative (Cummings *et al.*, 2001).

2.2.1 CONCEPT OF PREBIOTICS

The concept of prebiotic considers that many potentially health-promoting microorganisms are already resident in the human colon (Gibson and Roberfroid, 1995). To be an effective prebiotic the ingredient must:

i. Neither be hydrolyzed nor absorbed in the upper part of the gastrointestinal tract;
ii. Have a selective fermentation such that the composition of the large intestinal microbiota is altered towards a healthier composition (Wallace *et al.*, 2011).

2.2.2 NON-DIGESTIBLE OLIGOSACCHARIDES (NDO)

Oligosaccharides are carbohydrates with a low degree of polymersiation (DP) and therefore low molecular weight. They have been variously defined as including anything from 2 to 20 monosaccharide units (Cummings and Stephen, 2007). The main categories of non-digestible oligosaccharide presently available or in development as food ingredients include carbohydrates in which the monosaccharide unit is fructose, galactose, glucose, and/or xylose (Cummings and Stephen, 2007; Aida *et al.*, 2009). NDOs are readily water soluble and exhibit some sweetness, but solubility decreases with longer chain length (Angus *et al.*, 2005). Furthermore due to being undigested in the colon, they have caloric value but due to colonic fermentation, they have an energy contribution to food of about 1.5 kcal/g, similar to soluble fibre (Roberfroid and Slavin, 2000). Inulin and oligofructose have a specific chemical structure which our digestive enzymes cannot hydrolyse. Both substances are metabolised as dietary fibres in our body. Inulin and oligofructose also show beneficial dietary fibre effects such as a relief of constipation, increased

stool volume and an increased faecal acidity (Coussement, 1996). Inulin and oligofructose belong to a group of carbohydrates known as non-digestible oligosaccharides (NDOs), which are commonly consumed in a standard Western diet (Gibson *et al.*, 1994).

2.2.3 DESCRIPTION OF COMMON PREBIOTICS: OLIGOSACCHARIDES

Oligosaccharides are a group of short chain non-digestible polysaccharides consisting of between approximately 2 and 20 saccharide units, may be linear or branched, and occur in a wide variety of foods (Shin *et al.*, 2000; Cummings and Stephen, 2007). Fructo-oligosaccharides are widely distributed in plants such as onion, leek, asparagus, chicory, Jerusalem artichoke, garlic, wheat and oat as well as soybean asparagus and also made by the action of fructosyltransferase on sucrose (Plou *et al.*, 2002; Collins and Rastall, 2008). They are not hydrolysed by the human digestive enzymes, but are utilised by intestinal bacteria (Hidaka *et al.*, 1986; Kaplan and Hutkins, 2000). Oligosaccharides can be commercially produced through the hydrolysis of polysaccharides (e.g. dietary fibres, starch) or through enzymatic transfer reactions from lower molecular weight sugars (Mitsouka *et al.*, 1987; Plou *et al.*, 2002). The roles of some major prebiotics are stated in Table 2.2 (Adebola *et al.,* 2014; Azad, 2015; George *et al.,* 2016).

2.2.3.1 Lactulose

Lactulose is a synthetic disaccharide in the form Gal β1-4 fru (Gibson, 2004). Lactulose has been used as a laxative as it is not hydrolysed or absorbed in the small intestine (Gibson, 2004). Lactulose (4-O-β-D-galactopyranosyl-D-fructose), a keto analogue of lactose, serves as a substrate for preferential growth and increased development of some LAB (Desai, 2008). Lactulose is not metabolized by human or animal species and resists degradation by the lactases in the digestive tract (Cashman, 2002).

Table 2: Roles of Prebiotics

Prebiotic Factor	**Origin**	**Microbes Stimulated**	**Effects**
Oligosaccharides	Onion, garlic, chicory, root, burdock, asparagus, Jerusalem artichoke, soybean, wheat bran	*Bifidobacterium* species	Increase in bifidobacterium, suppression of putrefactive bacteria, prevention of constipation and diarrhoea
Frutooligosaccharides (inulin, oligofructo)	Onion, garlic, chicory, root, burdock, asparagus, Jerusalem artichoke, soybean, wheat bran	*Bifidobacterium* species, *Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus plantarum*	Growth of bifidobacteria and acid promotion.
Fructan	Ash-free white powder from tubers of Jerusalem artichoke	*Bifidobacterium* species	Growth of bifidobacteria
Human kappa casein and derived glycolmacropeptide	Human milk: chymotrypsin and pepsin hydrolysate	*Bifidobacterium bifidum*	Growth promotion
Stachyose and raffinose	Soybean extract	*Bifidobacterium* species	Growth factor
Casein macropeptide	Bovine milk	*Bifidobacterium* species	Growth promotion
Lactitol (4-O-β-D-galactopyranosyl) D-glucitol	Synthetic sugar alcohol of lactose	*Bifidobacterium* species	Growth promotion
Lactutose (4-O-β-D-galactopyranosyl) D-fructose	Synthetic derivative of lactose	*Bifidobacterium* species	Growth promotion

2.2.3.2 Inulin

Inulin is a blend of fructan chains found widely distributed in nature as plant storage carbohydrates (Wang and Gibson, 1993), and is present in more than 36,000 plant species. The majority of inulin commercially available today is extracted from chicory roots. Chemically speaking, inulin is a mixture of poly- and oligo-saccharides of which almost all have the chemical structure GFn (G =

glucose, F = fructose and n = number of fructose units linked to one another). The maximum amount of fructoses in inulin from chicory is about 60. The links between the molecules are of a very special type: the β(2-1) form, which makes these molecules indigestible for all higher animals. Inulin-type fructans contain both GFn (α D glucosyl- [β D fructosyl]n-1-D fructoside) and FFn (β D fructosyl-[β D fructosyl]n-1-D fructoside) molecules, with the number of fructose units varying from two to more than 70 units (Desai, 2008). The fructose units in the mixture of linear fructose polymers and oligomers are each linked by β-(2,1) bonds. A glucose molecule typically resides at the end of each fructose chain and is linked by an α-(1,2) bond, similar to sucrose. Chain lengths of these chicory fructans range from 2-60, with an average degree of polymerisation of 10 (Desai, 2008).

2.2.3.3 Isomalto-oligosaccharides

Isomalto-oligosaccharides exist in fermented foods such as soy, sauce, sake and honey.. Bifidobacteria and the *Bacteroides fragilis* group have been found to utilise isomalto-oligosaccharides, whereas *Escherichia coli* and other bacteria cannot (Macfarlane *et al.*, 2008).

2.2.3.4 Fructo-oligosaccharides (FOS)

Fructo-oligosaccharides (FOS) are structural relatives of inulin and are lower molecular weight versions. Two different types of fructo-oligosaccharides are common: inulin extracted from chicory roots which can be hydrolysed under controlled conditions by the enzyme inulinase to produce short-chain FOS represented as Glu-α1-2[β-D-Fru 1-2]n where n = 2-9 and FOS product known as `neosugar' or `meioligo' which is a mixture of three oligosaccharides of different lengths, i.e. 1-ketose (Glu-Fru2) and 1F -β-fructosylnystose (Glu-Fru4) (Rossi *et al.*, 2005). The mixture is enzymatically synthesised from sucrose by the transfructosylation action of β-fructosidase from the fungus "*Aspergillus niger*" (Goosen *et al.*, 2007). FOS are not degraded or

absorbed in the upper human gastrointestinal tract, as such, they enter the colon intact where they are susceptible to metabolism by the resident microbiota (Hidaka *et al.*, 1991). The β configuration of anomeric C-2 in fructose monomers, is thought to make FOS resistant to hydrolysis by human digestive enzymes which display a high degree of specificity for glycosidic linkages (Gibson *et al.*, 2000).

2.2.3.5 Galacto-oligosaccharides

Galacto-oligosaccharides are galactose-containing oligosaccharides of the form Glu α1- 4[β Gal 1-6]n where n = 2 to 5, and are produced from lactose syrup using the transgalactosylase activity of the enzyme β-galactosidase (Lamsal, 2012).

2.2.3.6 Soybean oligosaccharides

The predominant oligosaccharides in soybeans are the trisaccharide raffinose and the tetrasaccharide stachyose which are able to reach the colon (Krause *et al.*, 1994; Peterbauer and Richter, 2001).

2.2.3.7 Lactosucrose

Lactosucrose is produced from a mixture of lactose and sucrose using the enzyme β- fructosidase (Seki and Saito, 2012).

2.2.4 SELECTION OF PREBIOTICS

One of the biggest constraints in the development of prebiotics is the limited knowledge the structure-function relationships in the molecules involved (Korakli and Vogel, 2006). At the current time there is little information on the relative efficacy and selectivity of these molecule. A useful prebiotic would:

a. Have low dosage forms;

b. Be non-carcinogenic;

c. Have a low calorific value;

d. Have multifunctional properties;

e. Easily incorporated into food vehicles;

f. Exert good preservative and drying characteristics;

g. Target the distal colon (Preidis and Versalovic, 2009).

2.2.5 PROBIOTICS AND PREBIOTICS IN THE NIGERIAN CONTEXT

It is pertinent to mention that the idea of probiotics and prebiotics are not necessarily new to the African world. For instance, Nigerian fermented food products such as *Ogi, Kunnu* (pap), *Iru* (fermented locust beans), *Lafun* (fermented yam flour) and *Ogiri* (fermented melon seed) have been developed and consumed for centuries. Almost all of these have been found to contain probiotic organisms (Oyetayo and Oyetayo, 2005). In fact, many of these indigenous fermented foods have been shown to exert antioxidant properties (Oboh and Amusan, 2009), a feature common to quite a number of prebiotic and probiotic food products.

2.3 SORGHUM

2.3.1 ORIGIN OF SORGHUM BICOLOR

It is generally accepted that sorghum (*Sorghum bicolor*) originated in Ethiopia, between 5,000 and 7,000 years ago (Obilana, 1985; ICRISAT, 2005). The crop migrated westwards and subsequently diversified into more than 200 different varieties with varying colours world-wide (Ogbonna, 2011). From there, sorghum was distributed along the trade and shipping routes around Africa and the Middle East to India, China and the Far East (Doggett, 1970; Kimber, 2000; Ibeawuchi, 2007).

Sorghum is mostly cultivated in the arid and the semi-arid regions of the world and is the fifth most important cereal after wheat, maize, rice and barley in terms of production (Food and

Agricultural Organization, 2003; FAO, 2006). In 2011, Nigeria was ranked the highest producer of *Sorghum bicolor* in the world (FAO, 2011).

In sub-Saharan Africa, sorghum is especially significant for food security for world's poorest people (Adebayo and Needum, 2011) and comes second after maize in being a staple food (Dendy, 1995; Olaniyan, 2015). Sorghum is drought resistant by nature (Jordan and Sullivan, 1982; National Research Council, 1996; Dicko *et al*., 2006; Adeyanju *et al.,* 2015), which makes it particularly relevant to sub-Saharan Africa, as an estimated half a million families are susceptible to hunger due to drought (FAO, 2003). In addition, the ability to tolerate periods of water-logging and high temperatures makes sorghum an attractive crop for providing dietary energy and protein for people living in these regions (Okorocha, 2015).

2.3.2 SORGHUM CULTIVATION IN NIGERIA

Two of the best known sorghum species, *Sorghum vulgare* and *S. bicolor* (L.) Moench, locally called guinea corn, are the most extensively grown cereal grains in Nigeria (Aba, 2004). With an estimated annual production volume of 7.0 million tonnes (Obilana, 2005), Nigeria is the largest sorghum producer in the West African sub-region, accounting for about 71% of the total regional sorghum output. Globally, the country leads in sorghum production for human consumption and has risen from its fifth position in 1995 (FAO, 1995) to be the third largest sorghum producer in the world, after the USA and India, where more than 90% of their sorghum harvest is used as an animal feed (Obilana, 2005).

In Nigeria, sorghum is used as a raw material for lager beer brewing. Large-scale beer brewing with sorghum malts initially proved very difficult due to some apparently insurmountable biochemical problems (EtokAkpan and Palmer, 1990). This resulted in its use only as an adjunct

in the form of grits or whole grains (Palmer *et al.*, 1989), or better still, mashing raw sorghum with some commercial enzymes (Bajomo and Young, 1992; Agu and Palmer, 1998).

2.3.3 SORGHUM PROCESSING AND UTILIZATION

2.3.3.1 Processing

Sorghum is the most amenable cereal grain to different processing technologies including: primary, secondary and tertiary methods (Obilana, 2005). Primary processing involves: fermentation, malting, wet & dry milling, boiling, roasting and popping. Secondary processing involves; brewing, beverage & drinks production, baking and confectionery making, steaming, extrusion (for pastes & noodles), while tertiary processing involves: composite flours (mixing of cereal/cereal flours, cereal/legume flours, cereal/cassava flours), biofortification and chemical fortification with additives. The different processing levels and their technologies are achieved using different agro-industrial equipments and machinery. These result in diversified end-products for foods, feeds, beverages, alcoholic and nonalcoholic drinks.

2.3.3.2 Utilization

The uses of sorghum in Nigeria can be grouped into two: traditional and industrial. The traditional uses include a variety of traditional foods, beverages and drinks while its non-food traditional uses include: thatching of roofs and fencing of compounds. Sorghum consumption for food is mainly in the form of flour or paste processed into two main dishes: 'OGI' or 'AKAMU', a thin porridge and 'TUWO', a thick porridge. Other dishes that are sometimes made from sorghum include a number of deep fried snacks, steamed dumplings, etc (Obilana, 1981).

Sorghum foods are also high in minerals, vitamins and some essential amino acids which are further enhanced through biofortification thus, making them superior to other cereal foods. They contribute more energy and digestible protein in the diets of the majority of the people in the sub-

saharan regions than those obtained from root and tuber crops (Aba *et al.*, 2004). In addition, its polyphenol (mostly tannin) contents are used as antioxidants just as the slow digestibility of sorghum starch and protein makes its foods useful in diabetic treatments (Ognonna, 2008).

2.4 FERMENTATION

Fermentation of cereal based foods is a common practice in Africa for food preservation. It is a technology that is simple; home based and has fed millions of people (Achi and Ukuru, 2015).

Fermentation is generally used to describe the desirable biochemical changes brought about by microorganisms and/or enzymes on primary diets/food products (Nout and Motarjemi, 1997; Blandino *et al*., 2003). Enzymatic fermentations involve chemical reactions whereby the enzyme acts as a catalyst, such as occurs when starch is converted to dextrin by α-amylase. When fermentation involves microorganisms, these are either naturally present on the substrate or they may be deliberately added in the form of starter cultures (Nout and Motarjemi, 1997). In microbiological terms, fermentation is a type of energy-yielding microbial metabolism whereby organic substrates are incompletely oxidized, whereby organic carbohydrates act as electron acceptors (Adams, 1990).

The four main fermentation types are alcoholic, lactic acid, acetic acid and alkali based. Yeasts usually predominate in alcoholic fermentations, resulting in the production of alcohol, whereas bacteria such as *Acetobacter* species are responsible for fermentations where alcohol is converted to acetic acid in the presence of oxygen (Blandino *et al*., 2003). The production of foods such as Japanese *natto* from cooked soybeans, *dawadawa* from African locust beans and *ogiri* from melon seeds often involve some form of alkaline fermentation, with *Bacillus subtilis* being the dominant species (Wang and Fung, 1996). LAB mainly carry out lactic acid fermentation (Gardner *et al.*, 2001).

2.4.1 LACTIC ACID FERMENTATION

LAB are essential for fermentation of most plant-based products (Gardner *et al.*, 2001). In this regard, LAB play various roles that include the production of safe products through biopreservation as a result of bacterial antagonism (Oyewole, 1997; Soomro *et al*., 2002), enhancement of sensorial and nutritive value of the foods, and saving of energy through reduced cooking time (Simango, 1997). A plethora of LAB predominate in plant-based foods such as *pozol*, a Mexican maize dough (Escalante *et al*., 2001), Colombian *chicha* (Steinkraus, 1996), and *idli*, a fermented Asian food made from rice blended with black gram (Soni and Sandhu, 1991). LAB also play a central role during the production process of most fermented sorghum-based foods such as Ethiopian *injera* (Vogel *et al.*, 1993), Sudanese *kisra* (Mohammed *et al.*, 1991), Ugandan *bushera* (Muyanja *et al.*, 2003) and Tanzanian *togwa* (Mugula *et al.*, 2003). Metabolism of carbohydrate by LAB is shown in Figure 2.1 (Gänzle *et al.,* 2007).

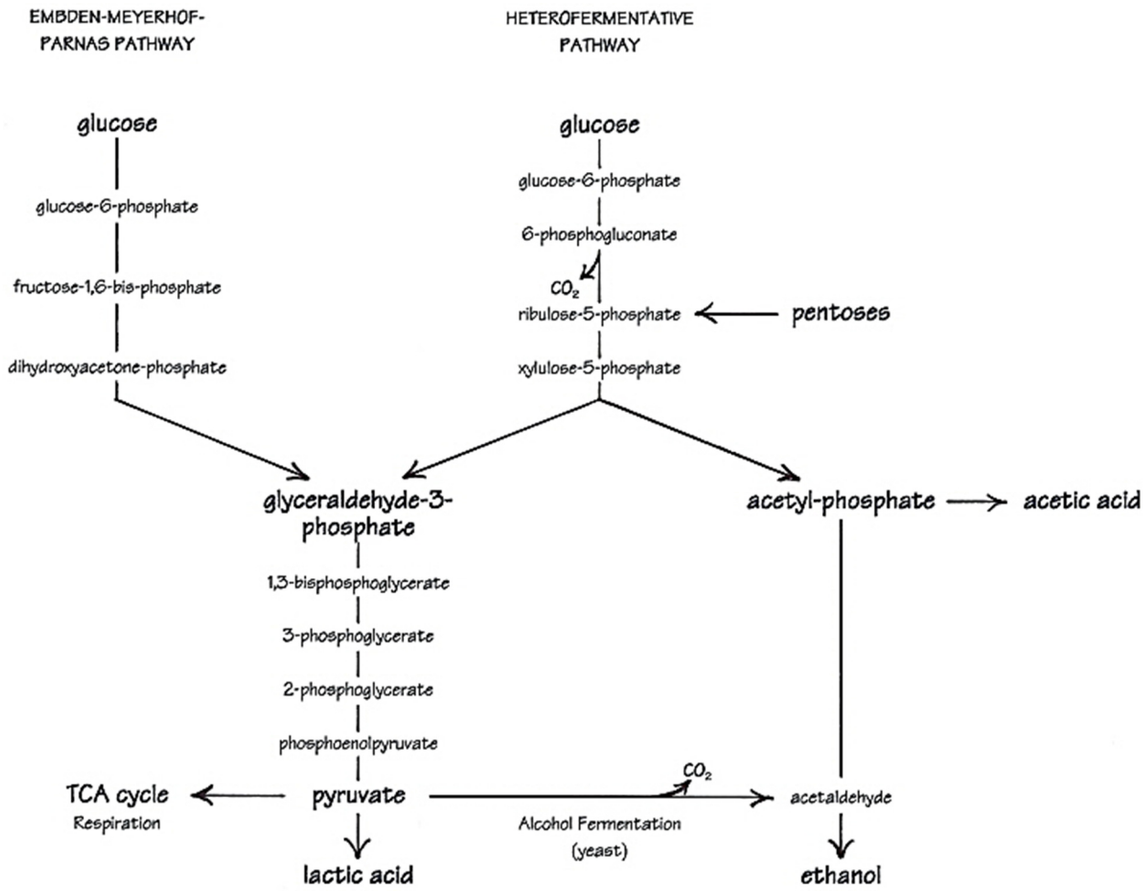

Figure 1: Diagrammatic illustration of metabolism of carbohydrate by lactic acid bacteria

2.4.2 NUTRITIONAL AND HEALTH ADVANTAGES OF LACTIC ACID FERMENTATION

Lactic acid fermentation of sorghum foods by LAB prior to cooking and/or consumption significantly alters their biochemical properties, rendering a food product with enhanced nutritional value and flavour (Towo *et al*., 2006). For instance, lactic acid fermentation improves the *in vitro* digestibility of starch (Hassan and El Tinay, 1995) and sorghum protein (Kazanas and Fields, 1981; Taylor and Taylor, 2002; Ibrahim *et al*., 2005). The increase in *in vitro* protein digestibility may be due to a rapid decrease in pH that affects the structure of insoluble sorghum proteins such as prolamines and glutelins in such a way that they become more accessible to pepsin

digestion (Taylor and Taylor, 2002). Lactic acid fermentation has been shown to reduce the level of anti-nutritive oligosaccharides, phytate and tannins. The amount of poly- and oligo-saccharides in sorghum, e.g., raffinose, stachyose and verbascose, are significantly reduced due to the action of β-galactosidases produced by some LAB that include *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Weissella paramesenteroides*, *Lactobacillus fermentum*, *Lb. brevis* and *Lb. buchneri* (Mital *et al*., 1973; Milliere *et al*., 1989). The β-galactosidases disrupt the β-D-galactosidic bonds of oligosaccharides, resulting in lowered abdominal distention and flatulence (Nout and Motarjemi, 1997). Tannin levels are also reduced by lactic acid fermentation (Lorri, 1993; Osman, 2004). The reduction may be due to metabolic processes of the microorganisms.

These processes include oxidation, reduction or dissociation of the tannins as a defense mechanism against toxicity by endogenous sorghum microflora (Bvochora *et al*., 2005). Reduced levels of phytate, as a result of lactic acid fermentation, may increase B vitamins (Kazanas and Fields, 1981; Nout and Motarjemi, 1997) and improve the availability of minerals (Marfo *et al*., 1990).

Some LAB have probiotic properties (Fuller, 1989). These include prevention of kidney stones (Campieri *et al*., 2001), treatment of heart disease (Schaafsma *et al*., 1998), control of cholesterol (Gilliland *et al*., 1985), stimulation of anti-carcinogenic action (Goldin, 1990), prevention of antibiotic-induced diarrhoea (Fooks *et al*., 1999), prevention of lactose intolerance (Gilliland and Kim, 1984), prevention of food allergies (Sutas *et al*., 1996), blockage of the formation of biogenic amines (Joosten and Nunez 1996) and prevention of vaginosis in humans (Reid, 2001).

For these reasons, thorough characterization of LAB for use in food fermentations is of utmost importance.

2.4.3 SENSORY ADVANTAGES OF LACTIC ACID FERMENTATION

During the production of many fermented foods, the development of appetizing flavour characteristics is crucial. Particularly, during cereal fermentations, several volatile compounds are formed, which contribute to a complex blend of appetizing flavours (Chavan and Kadam, 1989). The specific flavour occurs due to secondary metabolites produced during fermentation of macromolecules such as sugars (Longo and Sanromán, 2006). The specific flavour compounds produced include diacetyl, which imparts a buttery aroma (Davis *et al*., 1985), acetaldehyde (yoghurt flavour) (Abd El-Salam and Alichanidis, 2004) and the amino acid alanine, a natural sweetener (Hols *et al*., 1999). Generally, these flavour compounds are products of pyruvate, which acts as an electron or hydrogen acceptor during metabolism by some LAB and yeasts, thereby perpetuating fermentation through recycling of NAD+ (Axelsson, 2004).

Depending on the bacterial strain under investigation and the growth conditions, pyruvate may act as substrate for the following pathways: dehydrogenase pathway (aerobic metabolism), pyruvate oxidase pathway, pyruvate-formate lyase system (anaerobic) or the diacetyl/acetoin pathway (Caplice and Fitzgerald, 1999; Axelsson, 2004). When pyruvate is present in excess (compared to NAD+ regeneration), as occurs in the presence of another carbon source such as citrate, or if an alternative electron acceptor such as oxygen is present, the diacetyl/acetoin pathway is followed (Hugenholtz, 1993; Figure 1). The subsequent reactions are divided into three pathways, depending on the substrate, which interacts with the active acetaldehyde. Ultimately, pleasant sensory compounds, which include acetoin and diacetyl, are formed. Diacetyl formation usually occurs at low pH and decreased sugar concentration (Axelsson, 2004). Despite the pleasant sensory attributes imparted by these flavour compounds, high concentrations of diacetyl (above 5-7 mg/L) are considered unpleasant (Davis *et al*., 1985).

The pyruvate-formate lyase system is active under anaerobic conditions or when some substrate is limiting (Axelsson, 2004). This results in mixed acid fermentation wherein pyruvate and Coenzyme A (CoA) react to form formate and acetyl CoA with pyruvateformate lyase acting as the catalyst (Figure 1). The utilization of acetyl CoA results in the formation of ethanol (if it is used as an electron acceptor) or acetate (if it acts as a precursor for substrate-level phosphorylation) (Figure 1), or a mixture of both. *Lb. casei* and some *Lactococcus lactis* strains mainly use this pathway (Thomas *et al*., 1979). The pyruvate dehydrogenase pathway, which is mainly active in lactococci, involves pyruvate dehydrogenase that catalyses the formation of acetyl CoA under aerobic conditions in a manner similar to the pyruvate-formate lyase system, thus resulting in the formation of acetate (Figure 1). In the pyruvate oxidase pathway, pyruvate oxidase catalyses the conversion of pyruvate to acetyl-phosphate (acetyl-P) and carbon dioxide with the simultaneous formation of hydrogen peroxide. Ultimately, acetate is formed from acetyl-P (Figure 1). *Lb. plantarum* was reported to produce high concentrations of acetic acid, using pyruvate oxidase under aerobic conditions.

Despite the sensory advantages that LAB impart to fermented foods, these bacteria may, however, also produce off-flavours in beer and wine (Caplice and Fitzgerald 1999), and in fish (Lyhs *et al*., 2002). For instance, some strains of *Lb. plantarum* may cause off-flavours through the formation of aldehydes (Bottazi, 1988; Henick-Kling, 1995). Furthermore, the production of diacetyl by *Pediococcus damnosus* results in cloudy beer with an acid taste (Satokari *et al*., 2000). Some species of LAB (e.g., *L. kunkei*) and acetic acid bacteria (e.g., *A. aceti*) may also suppress yeast growth during grape juice fermentation, resulting in sluggish or stuck fermentations (Huang *et al*., 1996). This highlights the importance of accurate characterization of the LAB for use during specific fermentations.

2.4.4 SAFETY BENEFITS OF LACTIC ACID FERMENTATION

LAB render fermented foods safe for consumption by controlling the growth of pathogenic and spoilage microorganisms (Caplice and Fitzgerald, 1999). In order to create an unfavourable environment for these pathogenic and spoilage microorganisms, LAB produce a range of compounds that act as natural antimicrobials in different ways (Awojobi *et al.,* 2016). These compounds include, but are not limited to, bacteriocins, reutericyclin, diacetyl, hydrogen peroxide, carbon dioxide and organic acids such as lactic and acetic acid (De Vuyst and Vandamme, 1994; Leroy and De Vuyst, 2004). Some of these antimicrobial compounds were shown to be bactericidal or bacteristatic for a diverse range of microorganisms belonging to the following genera: *Streptococcus*, *Mycobacteria*, *Lactococcus*, *Listeria*, *Clostridium*, *Bacillus*, *Neiserria*, *Campylobacter*, and *Haemophilus* (Soomro *et al*., 2002; Savadago *et al*., 2006).

Some of the antimicrobial agents, e.g., bacteriocins, organic acids and carbon dioxide, produced by LAB disrupt the cytoplasmic membranes of target organisms by virtue of different properties (Riley and Wertz, 2002). For example, lactococcin A depolarizes or makes pores in the cytoplasmic membrane, thus disrupting the integrity of this structure (Van Belkum *et al*., 1991). Reutericyclin is a highly hydrophobic and charged molecule that is produced during anaerobic growth of *Lactobacillus reuteri* (Ganzle and Vogel, 2003). This compound is bacteriostatic or bactericidal against a broad range of food-associated spoilage and pathogenic microbes, including *Staphylococcus aureus* and *Listeria innocua*, due to its activity as a proton ionophore, resulting in translocation of protons across the cytoplasmic membrane and consequently, dissipating the transmembrane pH gradient (Ganzle, 2004).

2.5 FERMENTED FOODS

Campbell-Platt (1987) has defined fermented foods as those foods which have been subjected to the action of micro-organisms or enzymes so that desirable biochemical changes cause significant modification to the food.

Fermented cereal foods play an important socio–economic role in developing countries as well as making a major contribution to the protein requirements of natural populations (Achi and Ukuru, 2015).

Cereals are a major component of human food in Africa. Nutritional experts have paid attention to cereal based foods from maize, sorghum and millet sources. These cereals have high content of soluble non-starch polysaccharides such as beta glucan which has a health promoting role.Consequently, foods submitted to the influence of lactic acid producing microorganisms is considered a fermented food (Odunfa and Adeyele, 1985).

2.5.1 CLASSIFICATION OF FERMENTED FOODS

Fermented foods can be classified in many different ways. The classification often is according to the kind of microorganism involved (Yokotsuka, 1982). Other classifications are based on commodity (Kuboye, 1985; Campbell-Platt, 1987; Odunfa, 1988;). Also, fermented foods classification can be based on the function of the food (substrate) (Odunfa and Adeyele, 1985; Caplice and Fitzgerald, 1999).

2.5.2 SORGHUM-BASED FERMENTED FOODS

Throughout the world, a plethora of fermented foods are metabolized by a variety of microorganisms to yield products with unique and appealing characteristics (Caplice and Fitzgerald, 1999; Taylor, 2003). In Africa, sorghum-based fermented foods and beverages include

injera (Chavan and Kadam, 1989), *kisra* (Mohammed *et al*., 1991), *ogi* (Akingbala *et al*., 1981), *mahewu* (Bvochora *et al*., 1999), *uji* (Mbugua *et al*., 1984), *muramba* (Mukuru, 1992), *bushera* (Muyanja *et al.,* 2003), *togwa* (Lorri and Svanberg, 1995) and *ting* (Boling and Eisener, 1982). LAB are mainly associated with most of these fermented foods (Table 3) (Ali, 2010).

Table 3: Fermented foods and beverages and their associated lactic acid bacteria

Types of fermented products	Lactic acid bacteria
Dairy products	
Hard cheeses without eyes	*L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris*
Cheeses with small eyes	*L. lactis* subsp. *lactis*, *L. lactis* subsp. *lactis* var. *diacetylactis*, *L. lactis* subsp. *cremoris*, *Leuc. mesenteroides* subsp. *cremoris*
Swiss- and Italian-type cheeses	*Lb. delbrueckii* subsp. *lactis*, *Lb. helveticus*, *Lb. casei*, *Lb. delbrueckii* subsp. *bulgaricus*, *S. thermophilus*
Butter and buttermilk	*L. lactis* subsp. *lactis*, *L. lactis* subsp. *lactis* var. *diacetylactis*, *L. lactis* subsp. *cremoris*, *Leuc. mesenteroides* subsp. *cremoris*
Yoghurt	*Lb. delbrueckii* subsp. *bulgaricus*, *S. thermophilus*
Fermented, probiotic milk	*Lb. casei*, *Lb. acidophilus*, *Lb. rhamnosus*, *Lb. johnsonii*, *B. lactis*, *B. bifidum*, *B. breve*
Kefir	*Lb. kefir*, *Lb. kefiranofacies*, *Lb. brevis*
Fermented meats	
Fermented sausage (Europe)	*Lb. sakei*, *Lb. curvatus*
Fermented sausage (USA)	*P. acidilactici*, *P. pentosaceus*
Fermented fish products	*Lb. alimentarius*, *C. piscicola*
Fermented vegetables	
Sauerkraut	*Leuc. mesenteroides*, *Lb. plantarum*, *P. acidilactici*
Pickles	*Leuc. mesenteroides*, *P. cerevisiae*, *Lb. brevis*, *Lb. plantarum*
Fermented olives	*Leuc. mesenteroides*, *Lb. pentosus*, *Lb. plantarum*, *P. acidilactici*, *P. pentosaceus*, *Lb. plantarum*, *Lb. fermentum*
Fermented vegetables soy sauce	*T. halophilus*
Fermented cereals	
Sourdough	*Lb. sanfransiscensis*, *Lb. farciminis*, *Lb. fermentum*, *Lb. brevis*, *Lb. plantarum*, *Lb. amylovorus*, *Lb. reuteri*, *Lb. pontis*, *Lb. panis*, *Lb. alimentarius*, *W. cibaria*
Alcoholic beverages	
Wine (malolactic fermentation)	*O. oeni*
Rice wine	*Lb. sakei*

B = *Bifidobacterium*; C = *Carnobacterium*; L = *Lactococcus*; Lb = *Lactobacillus*;
Leuc = *Leuconostoc*; O = *Oenococcus*; P = *Pediococcus*; S = *Streptococcus*;
T = *Tetragenococcus*; W = *Weissella*

2.5.2.1 *Ogi*

Ogi, a fermented sorghum, maize or millet gruel, is considered the most significant weaning food in West Africa and has been produced on a semi-industrial scale (Olukoya *et al.,* 1994; Achi,

2005). *Ogi* has a sour flavour akin to that of yoghurt and a characteristic aroma, which differentiates it from other cereal-based fermented foods. Briefly, *ogi* is prepared by steeping the cereal grains in pots for 1-3 days, followed by wet milling and sieving of the fermented grains to yield a slurry of which the colour depends on the cereal grain (Odunfa, 1985). Fermentation of the grains is steered by LAB, yeasts and moulds, whilst flavour development is imparted by members of the genera *Saccharomyces* and *Candida* (Caplice and Fitzgerald, 1999).

The major organisms responsible for the fermentation and nutritional improvement of *Ogi* include *Lactobacillus plantarum*, *Corynebacterium, Aerobacter*, the yeasts *Candida, Mycoderma, Saccharomyces cerevisiae* and *Rhodotorula*. Others include moulds like *Cephalosporium, Fusarium, Aspergillus* and *Penicillium* species (Achi and Ukuru, 2015). Odunfa, (1985) observed that *L. plantarum* was the predominant organism in the fermentation responsible for lactic acid production. *Corynebacterium* hydrolysed corn starch to organic acids while *S. cerevisiae* and *Candida mycoderma* contributed to flavour development.

2.5.2.2 *Pito*

Pito is an indigenous Nigeria alcoholic drink produced from local grains such as guinea corn (*Sorghum vulgare*) and sorghum (*Sorghum bicolor*). Sorghum is one of the cereals cultivated in the tropical regions of sub-Saharan Africa. It is largely cultivated in northern Nigeria (Asiedu 1989).The nutritional composition of sorghum include starch 68-80 % , protein 10-15% moisture content 11- 12% , fat 3%, fibre 2% and ash content 2% (Ihekoronye and Ngoddy 1985). The indigenous alcoholic drink pito is produced locally by malting of the sorghum grains, fermentation and maturation (incubation period) (Ekundayo 1969). The microbial organisms associated with the fermentation of this indigenous alcoholic drink include *Saccharomyces cerevisae*, *Saccharomyces chavelieria* and *Leuconostoc meseteroides*. The local method of pito production is

similar to methods used to produce other local alcoholic drinks like burukutu except increased pH from 4.2 - 5.2 within 24 hr of fermentation and sharp decrease of pH to 3.7 after 48 hr (Asiedu 1989). *Geotrichum candidum*, and *Lactobacillus* species have been said to be responsible for souring pito. Another type is unfermented pito is available which is also an indigenous product produced in Ilorin, Nigeria mainly from plant extracts. The process of producing unfermented pito involues steeping, and boiling only. Kolawale *et al*. (2007) have reported the chemical composition and microbial quality of pito. It has been observed that there was a variation in the nutrient content of pito produced using varying processing methods .The protein content of fermented and unfermented pito showed 2.5% and undetected level respectively. Most of the bacterial cultures found during the production of pito include lactic acid bacteria, which include those bacteria capable of metabolising lactose to lactic acid that lowers pH of product. The lactic acid bacteria have optimum pH 3 - 6.8. Many lactic acid bacteria have been found to act as bacteriocin and have been in fermented pito. They include *Leuconostoc mesenteriod, Bacillus subtitis, Staphylococcus* species, *Geotrichum candidum* and *Lactobacillus* species. The species are responsible for the souring of pito (Okoro *et al.,* 2011). *Pito* is an excellent source of calories and also contributes valuable protein to consumers (Ekundayo, 1969).

2.5.2.3 *Injera*

Injera is a circular, spongy-textured Ethiopian sour bread (Stewart and Getachew, 1962; Chavan and Kadam, 1989) made from sorghum, tef, corn, finger millet or barley. Over 8% of the total sorghum production in Ethiopia is used for *injera* production (Gebrekidan and Gebrettiwat, 1982). The production process for *injera* involves mixing of the mechanically dehulled grains with water, followed by addition of the starter (*ersho*) from a previous batch and fermentation for 2-3 days. A small part of the fermented mixture is gelatinized in boiling water, added back to the fermented

dough and allowed to stand for 2-3 h. The resulting dough is thinned down after fermentation and poured into a thinly oiled pan, covered with a lid for 2-3 min and cooked (Parker *et al*., 1989). This food is valued for its sour flavour and high nutritional value, being rich in calcium and iron (Zegeye, 1997). Yeasts and some fungi are mainly involved in the fermentation of *injera* (Blandino *et al*., 2003).

2.5.2.4 *Kisra*

Fermented sorghum is used to make *kisra* that is baked into thin sheets to make pancakes, which are consumed throughout the Arabian Gulf, Sudan and Iraq (Eggum *et al*., 1983). *Kisra* pancakes are similar to *injera*. The fermented dough (*ajin*) is prepared by using a portion of a previous batch (back-slopping). Fermentation was found to increase the crude protein, thiamine and niacin content of *kisra* (El Tinay *et al*., 1979; Axtell *et al*., 1981; Eggum *et al*., 1983). A range of LAB, yeasts and fungi are involved in the production of *kisra*.

2.5.2.5 *Ting*

In South Africa and Botswana, *ting* is used as a weaning food and is also consumed by adults (Boling and Eisener, 1982). This food represents a fermented sour porridge made traditionally by soaking sorghum flour for 2-3 days in water, followed by cooking. *Ting* is highly prized by local people for its unique taste, texture and aroma. Despite its popularity, there is little information regarding the microorganisms associated with the production of *Ting* (Boling and Eisener, 1982).

2.5.2.6 *Togwa*

Togwa, a Tanzanian fermented gruel or beverage, is produced from sorghum, millet, maize, cassava or combinations of these (Mugula *et al*., 2001). It is useful as a weaning food or as a refreshment (Mugula *et al*., 2001). Like other indigenous African foods, the predominant

microflora are LAB and yeasts (Table 3). The consumption of *togwa* is declining, probably due to the unhygienic nature of the preparation method and its poor shelf-life (Mugula *et al*., 2001).

2.5.2.7 *Bushera*

Bushera, a non-alcoholic fermented beverage, is the most common indigenous beverage produced in the western highlands of Uganda (Muyanja *et al*., 2003). Production of *bushera* involves mixing germinated sorghum flour with boiled water, followed by cooling at ambient temperature and addition of germinated sorghum flour for starting the fermentation (Muyanja *et al*., 2003). The fermentation is dominated by LAB and normally takes place in clay pots for 1-4 days (Muyanja *et al*., 2003).

2.6 STARTER CULTURES

Starter cultures are microbial preparations containing large numbers of cells with at least one type of microbe, which shorten and steer the fermentation process when added to the raw material (Leroy and De Vuyst, 2004). These cultures are critical for the formation of the desired flavour compounds that contribute to specific sensory characteristics.

Using amylolytic lactic acid bacteria as starter culture offers an alternative by combining both amylase production and acidification in one microorganism (Santoyo *et al.,* 2003). Pure yeast cultures have been shown to increase proteincontent of fermented cereal products. Lactobacilli are the predominant organisms involved in the fermentation of cereal based foods and beverages in Africa. Beneficial starter cultures are not usually used in traditional fermentation of cereal based products. The fermented foods have probiotic potentials due to the *Lactobacillus* species contained in them. There are reports of the quality of some African fermented product that have been enhanced using beneficial cultures (Nyanzi and Jooste, 2013). *Ogi* was enhanced with a lactic acid starter culture and the product was called Dogik. The starter cultures have antimicrobial activities

against diarrhoeagenic bacteria. Onyango *et al*, (2004) also reported the presence of *Lactotobacillus paracasei,* a probiotic *Lactobacillus* in association with other LABs in *Uji* – a cereal fermented food. Nout, (1991) reported that *Lactobacillus acidophilus* which are probiotic were isolated from African fermented sorghum – based products. Nwachukwu *et al,* (2010) have studied the spontaneous fermentation of cereals (Maize, millet, sorghum) for the production of *Ogi* and *kunun–zaki*. They reported the presence of lactic acid bacteria such as *Lactobacillus plantarum, L. pentosus, L. celboiosus, Pedicoccus pentosaceus* and *Leuconostoc mesenteroides*. *L. plantarum* was shown to have potential of being a starter culture for the fermentation of maize for *Ogi* production (Achi and Ukuru, 2015).

2.6.1 LACTIC ACID BACTERIA (LAB)

Taxonomy, a synonym of biosystematics, involves the cataloguing of biodiversity with the aim of arranging and characterizing organisms in an organized way (Staley and Kreig, 1984). It is divided into classification, nomenclature and identification (Vandamme *et al*., 1996).

Classification is the orderly arrangement or clustering of organisms into taxonomic groups based on similarity, whilst nomenclature is the labeling of units according to international rules, and identification of organisms is the process of determining whether a query isolate belongs to one of the defined units (Staley and Kreig, 1989; Vandamme *et al*., 1996). The species is the most significant and central element of bacterial taxonomy (Vandamme *et al*., 1996) and has been described as a cluster of separate organisms, which are monophyletic, genomically coherent, and have a high degree of overall similarity with respect to independent characteristics (Rosselló-Mora and Amann, 2001). Bacterial species are usually identified using phenotypic traits, phylogenetic data or combinations of these.

Phenotypic classification usually involves carbohydrate fermentation patterns, lactic acid configuration and other growth requirements (Sharpe, 1981). Based on carbohydrate fermentation patterns, lactobacilli are classified as:

(i) obligately homofermentative;

(ii) facultatively heterofermentative;

(iii) obligately heterofermentative.

The differences between the three fermentation types are as follow: obligate homofermenters use the Emden-Meyerhof-Panas (EMP) pathway to convert hexose sugar to almost solely lactic acid, but not pentoses and gluconate due to the lack of phosphoketolase; facultative heterofermenters possess both aldolase and phosphoketolase, and can thus metabolize pentoses and gluconate in addition to hexoses, which are degraded using the EMP pathway; obligate heterofermenters metabolize pentoses, and hexoses are degraded via the phosphogluconate pathway with the formation of lactate, carbon dioxide, ethanol or acetic acid (Hammes and Vogel, 1995). Other phenotypic data may be obtained from the following: (i) expressed characteristics such as morphology, physiology, enzymology and serology, (ii) chemotaxonomic markers, which include cellular fatty acids and exopolysaccharides, among others, and (iii) proteins, including functionality (Vandamme *et al*., 1996).

The classification of LAB, which is based on phenotypic traits and true phylogenetic relationships inferred from rRNA gene sequences, remains largely unresolved due to lack of correlation between these approaches (Vandamme *et al*., 1996; Felis and Dellaglio, 2007). For instance, the genus *Lactobacillus* is phylogenetically intermixed with some *Pediococcus* and *Leuconostoc* strains, despite their traditional separation due to differences in morphology (Stackebrandt and Teuber, 1988; Axelsson, 2004). In addition, phenotypic tests mostly fail to distinguish the genus

Carnobacterium from that of *Lactobacillus*, and *Vagococcus* from *Lactococcus* members (Axelsson, 2004). Despite this lack of correlation in some groups between phenotypic and genotypic results, phenotyping generally provides important information for separating taxa (Botina *et al*., 2006). In order to circumvent limitations inherent in either phenotyping or phylogeny, polyphasic taxonomy, involving an integration of phenotypic, genotypic and phylogenetic data, is important (Colwell, 1970; Vandamme *et al*., 1996). However, if the main thrust is speed and reliability, DNA-based techniques are more advantageous (Ehrmann and Vogel, 2005).

2.6.2 GENERAL CHARACTERISTICS OF LACTIC ACID BACTERIA

LAB represent a diverse and paraphyletic group of bacteria. They constitute a heterogeneous group of Gram-positive, acid-tolerant and strictly fermentative cocci, coccobacilli or rods that produce lactic acid as the main product during carbohydrate fermentation (Axelsson, 2004; Temmermann *et al*., 2004; Ehrmann and Vogel, 2005). The heterogeneity is clearly evidenced in the genus *Weissella* that contains both coccoid and rodshaped members (Collins *et al*., 1993). Furthermore, the largest and most heterogeneous group within the LAB group comprise lactobacilli with a G + C content of 32-55%, which is wider than that expected for members of a single genus (Goodfellow *et al*., 1997). Generally, the G + C content do not vary more than 3 and 10% for a well-defined species and genus level, respectively (Stackebrandt and Liesack, 1993).

LAB generally lack catalase, although pseudocatalase was detected in cultures grown at low sugar concentrations (Caplice and Fitzgerald, 1999). They are asporogenous and fastidious organisms and hence, occupy nutritionally rich habitats. LAB are mesophilic and their growth temperatures range from 5°C to 45°C (Caplice and Fitzgerald, 1999). This variability also applies to pH where

LAB grow at pH 9.6 and pH 3.6, with the majority growing at pH 4.0-4.5 (Caplice and Fitzgerald, 1999).

2.6.3 THE ORDER LACTOBACILLALES

The order *Lactobacillales* belongs to the phylym Firmicutes, class Bacilli and comprises the LAB. Together with the *Lactobacillaceae*, this order includes five other families, as well as a number of species not currently classified in any known family (Garrity *et al*., 2001). The total number of genera within these families is 34 (Garrity *et al*. 2004), with the following being important fermenters or spoilers of fermented products: *Lactobacillus*, *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* (Axelsson, 2004).

The family *Lactobacillaceae* consists of three genera, namely *Lactobacillus*, *Paralactobacillus* and *Pediococcus* (Garitty *et al*., 2004). The genus *Lactobacillus* currently contains 106 species, making it the largest group in the order (Felis and Delaglio, 2007). The genus *Pediococcus* contains 11 members. The genus *Paralactobacillus* contains only one species, *Paralactobacillus selangolensis* (Leisner *et al*., 2000), which was isolated from *chili bo*, a Malaysian food ingredient (Leisner *et al.*, 2000).

The family *Leuconostoccaceae* consists of three genera, namely *Leuconostoc*, *Oenococcus* and *Weissella*. *Leuconostoc*, the largest of these, has 23 members and some of them are associated with food (Shaw and Harding, 1989; Antunes *et al*., 2002; Kim *et al*., 2003; Chambel *et al*., 2006). *Weissella*, the second largest genus in this family, has 12 members. Although more members of this genus have been isolated from fermented food (Collins *et al*., 1993; Choi *et al*., 2002), some *Weissella* species have been detected in clinical samples (Björkroth *et al*., 2002; Shin *et al*., 2007). The genus *Oenococcus* consists of only two members, *Oenococcus oeni* and *O. kitaharae*.

The genera *Streptococcus* and *Lactococcus* make up the family *Streptococcaceae*. Of the 97 *Streptococcus* members, *Streptococcus thermophilus* is of industrial significance and it is used during the manufacture of a variety of fermented milk products.

Most of the members in these genera are associated with a variety of diseases, both in humans and animals (Smith and Sherman, 1939; Bouvet *et al*., 1989; Whiley *et al*., 1999). The genus *Lactococcus* has six members with *Lactococcus lactis* playing a central role in the industrial production of various compounds such as nisin and diacetyl.

Enterococcus, *Atopobacter*, *Mellisococcus*, *Tetragenococcus* and *Vagococcus* genera belong to the family *Enterococcaceae* (Garrity *et al*., 2004). The largest of these genera *Enterococcus* consists of 37 members that are associated with both foods and clinical samples (Fortina *et al*., 2004; Koort *et al*., 2004). The remaining genera consist of a total of eight members (Garrity *et al*., 2004).

The family *Aerococcaceae* consists of seven genera (Garrity *et al*., 2004). The largest genus is *Facklamia*, which consists of six species. The genera *Abiotrophia* and *Aerococcus* have five members each. *Globicatella* genus has two members, whilst *Dolosicoccus*, *Eremococcus* and *Ignavigranum* consist of only one member per genus (Garrity *et al*., 2004).

The family *Carnobacteriaceae* consists of 12 genera (Garrity *et a*l., 2004), with a total of 24 members. The largest genus *Carnobacterium* consists of eight members, whilst the remainder contains one or two members only. Some members of *Carnobacteria* are associated with food spoilage (Holley *et al*., 2002).

2.6.4 IDENTIFICATION OF LAB ASSOCIATED WITH FERMENTED FOODS

2.6.4.1 Phenotypic Methods

Phenotyping of bacteria involves all the identification methods that are not based on DNA or RNA (Caplice and Fitzgerald, 1999). These include morphology (e.g., shape, presence or absence of endospores or inclusion bodies), physiology and biochemical attributes such as conditions required for growth, activities of enzymes and metabolic activities. The significance of individual phenotypic characteristics may appear insignificant for determining genetic relatedness, yet, when taken together, taxa may be determined using these attributes (Vandamme *et al*., 1996).

For routine phenotyping, miniaturized identification kits such as API 50 CHL or BIOLOG, which are based on carbohydrate fermentation profiles, are often popular due to affordability (Temmerman *et al*., 2004). Although this approach is simple, affordable and provides evidence for functionality of strains, phenotyping is generally laborious, time-consuming and the efficiency and resolution may be poor, especially when complex samples with high diversity are studied (Meroth *et al*., 2003). For instance, most LAB isolates from freshwater fish could not be identified, even at genus level, due to the poor taxonomic resolution of phenotypic methods (Gonzalez *et al*., 2000).

In addition, only 38% of LAB from sourdough could be identified using the API 50 CHL system, as well as morphological and physiological characteristics (Corsetti *et al*. 2001). The fact that LAB strongly adapt to their environment and share many common attributes, makes differentiation of species using phenotypic methods challenging (Hayford *et al*., 1999; Van Reenen and Dicks, 1996). Moreover, it is challenging to compare results obtained using different phenotypic methods and similar phenotypes may not be concordant with related genotypes (Temmerman *et al*., 2004).

Therefore, it is essential to use an approach that combines phenotyping and fast, reliable and reproducible DNA-based techniques with high resolution for identification of LAB associated with food fermentations (Vandamme *et al*., 1996; Nigatu, 2000).

2.6.4.2 Genotypic Characterizations

LAB are characterized using a wide variety of DNA-based methods. One DNAbased method that will allow identification of LAB to the species level is species-specific
PCR (Nomura *et al*., 2002). Species-specific PCR involves the amplification of target genes, using primers that correspond to the oligonucleotide sequences present within parts of the whole genome of target organisms (Lupski and Weinstock, 1992), followed by subsequent analysis. Species-specific PCR targeting the 16S rRNA gene was used for identification of *Lb. brevis* and the method was found to be very sensitive, specific and efficient (Guarneri *et al*., 2001).

DNA-based methods that allow identification of LAB to the species and strain level include restriction fragment length polymorphism (RFLP) (Giraffa *et al*., 2003), pulsed-field gel electrophoresis (PFGE) (Ventura and Zink, 2002), randomly amplified polymorphic DNA-PCR (RAPD-PCR) (Williams *et al*., 1990), repetitive extragenic palindromic-PCR (rep-PCR) (Versalovic *et al*., 1991), amplified ribosomal DNA restriction analysis (ARDRA), amplified fragment length polymorphism (AFLP) (Vos *et al*. 1995) and phylogenetic analyses of housekeeping loci.

RFLP involves restriction enzyme digestion of the whole-genome, followed by size fractionation of the resultant DNA fragments by agarose gel electrophoresis. Southern blotting (Southern, 1975) may be used to transfer DNA fragments onto either a nitrocellulose or nylon membrane, followed by hybridization to a labeled homologous probe (e.g., rRNA gene probe), which allows visualization of the hybridized fragments (Vandamme *et al*., 1996).

In ARDRA, bacterial rRNA genes are initially amplified by PCR using conserved sequences of rRNA genes as primers and the amplicon is digested with restriction endonucleases, followed by electrophoresis to obtain a fingerprint (Massol-Deya *et al*., 1995). ARDRA is fast to perform, but highly dependent on the degree of polymorphism in the region studied and the discriminatory power is often inferior (Massol-Deya *et al*., 1995) because smaller areas of the rRNA operon are targeted. Roy *et al*. (2001) used ARDRA for molecular discrimination of *Lb. helveticus*, *Lb. delbrueckii* subspecies *delbrueckii*, *Lb. delbrueckii* subsp. *lactis*, *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. acidophilus* and *Lb. casei*, making it a suitable technique for discrimination of these closely related bacteria at subspecies level (Roy *et al*., 2001).

PFGE involves separation of large DNA representing the whole genome in an oscillating electric field (Tenover *et al*., 1995; McCartney, 2002). Excellent subspecies discrimination is the hallmark of PFGE. For instance, Ventura and Zink (2002) observed heterogeneity among isolates of *Lb. johnsonii* after using PFGE, despite the close relationship between these organisms. However, since the extraction of intact chromosomal DNA is critical to PFGE, the technique becomes labourious and expensive. Therefore, PFGE it is seldomly applied in studies where large numbers of isolates are to be characterized (Temmerman *et al*., 2004).

In RAPD-PCR fingerprinting, one or two primers (usually 10-12 bp) are arbitrarily selected and allowed to anneal to the bacterial genomic DNA template at low stringency, resulting in several amplification products of varying sizes, which are resolved electrophoretically to yield a RAPD fingerprint (Welsh and McClelland, 1990). RAPD analyses do not require prior knowledge of the target sequences. It has been used to follow unmarked starter cultures in commercial fermentations (Plengvidhya *et al*., 2004). Reguant and Bordons (2003) developed a multiplex RAPD-PCR, based

on the combination of one random 10-mer and one specific 23-mer oligonucleotide in a single PCR.

Microorganisms are still described only as closely related even if they share identical rRNA sequences. Therefore, bacterial species are increasingly defined using multilocus sequence analysis (MLSA), involving housekeeping loci that evolve more rapidly than 16S rRNA genes (Gevers *et al.*, 2005).

2.6.4.3 Culturing Bacteria

Most aerobes and facultative anaerobes can be cultured relatively easily on commercially available highly selective media (Lee, 2014). Although the overall count of the organization of easily recognized groups such as 'coliforms', staphylococci, streptococci, yeast and lactobacilli. Few media of this sort are available for strict anaerobes. Almost all the bacteria that grow in the intestine are non-sporulating anaerobes. Thus, Escherichia coli, usually thought of as a typical faecal organism, constitute less than 1% of the bacteria in faeces. Many of the bacteria are extremely sensitive to oxygen, especially on initial isolation, and thus the usual techniques for the study of anaerobes from clinical sources are not ideal (Harrigan and McCance, 2014).

2.6.5 TAXONOMY OF LAB

The classification of LAB is still under investigation and will probably remain an important topic for taxonomists for quite some time. LAB comprise many genera including *Lactobacillus, Enterococcus, Streptococcus, Pediococcus* and *Leuconostoc* (Felis and Dellaglio, 2007).

2.6.5.1 The genus *Lactobacillus*

Lactobacillus is the most important genus, to which sixty four species belong. It was proposed by Beijer-inck in 1901. In 1974, Rogosa divided the Lactobacillus genus into three major groups.

Group 1 are the obligate homofermentative lactobacilli, degrading hexose almost exclusively into lactic acid and not capable of using pentoses or gluconate. The most important species in this group is *Lactobacillus acidophilus*.

Group 2 are the facultative heterofermentative lactobacilli, such as Lactobacillus plantarum. This species can either ferment hexoses almost exclusively into lactic acid or lactic acid, acetic acid, ethanol and formic acid.

Group 3 are the obligate heterofermentative gas forming lactobacilli, which are capable of fermenting hexoses into lactic acid, acetic acid, ethanol and carbon dioxide. As well as fermenting pentoses to lactic acid and acetic acid. A member of this group is Lactobacillus bifermentas (Rogosa, 1974).

2.6.5.2 The genus *Enterococcus*

The history of enterococci starts in 1899 with the description by Thiercelin of a new gram positive Diplococcus, which was later included in the new genus Enterococcus. It was classified as Group D Streptococcus until 1984 when genomic DNA analysis indicated that it's considered as a separate genus (Wood and Holzapfel, 1995). The members of this group are Gram positive, microaerophilic cocci, which are not motile and arranged inpairs or chains. They are difficult to be distinguished from streptococci on physical characteristics only, it can be defined by combination of antigenic, haemolytic and physiological characteristics (Wood and Holzapfel, 1995; Fischetti *et al.*, 2006).

2.6.5.3 The genus *Streptococcus*

Identification and classification of streptococci relied for a very long time on the serological groups introduced by Lancefield (1933). Sherman (1937) devided the streptococi into four groups which included the "enterococci", the "lactic streptococci", the "viridians" and the "pyogenic streptococci". After recent taxonomic changes the genus streptococci was reserved for nonspore

forming, cocci or coccobacilli, chemoorganotrophic microorganisms, arranged in pairs and chains. They ferment carbohydrates into lactic acid as the major end product, and are generally aerotolerant (De Vuyst and Vandamme, 1994; Wood and Holzapfel, 1995).

2.6.5.4 The genus *Pediococcus*

Until 1990, the genus Pediococcus comprised eight species, the oldest, *Pediococcus acidilactici*, have been described in 1887 (De Vuyst and Vandamme, 1994), the most recent one, *Pediococcus inopinatus*, in 1978 (Back and Stackebrandt, 1978). The genus *Leuconostoc* comprises 10 species, the oldest, Leuconostoc mesenteroides, being described as early as 1878 by Tsenkovskii, followed by *Leuconostoc dextranicum* and *Leuconostoc cremoris* (Tsenkovskii, 1878). The latter two species are now considered to be subspecies of *Leuconostoc mesenteroids* (De Vuyst and Vandamme, 1994). Members of this genus are heterofermentative cocci arranged in pairs or short chains (Schillinger and Lucke, 1989). *Pedioccocus* spp. are widely distributed in fermenting planting material (Akinkugbe and Onilude, 2013).

2.6.6 PRODUCTION OF ANTIMICROBIAL SUBSTANCES BY LAB

Antimicrobial substances produced by lactic acid bacteria can be divided into two main groups: low molecular mass substances with molecular mass 1,000 Da, such as bacteriocins (Ogunbanwo *et al.,* 2003). All non-bacteriocin antimicrobial substances from LAB are of low molecular mass. Low molecular mass antimicrobials. The metabolites of LAB with antimicrobial activity are accumulated in their environment at the levels and proportions that depend on the species of LAB and chemical composition of the growth media. Fermentation of hexoses by lactic acid bacteria is characterized by homofermentative production of lactic acid or by heterofermentative production of equimolar amounts of lactate, acetate/ethanol and carbon dioxide. Pentoses are fermented by many heterofermentative and homofermentative LAB in the same way since phosphoketolase of

homofermentative LAB is generally inducible by pentoses. Fermentation of pentoses yields the equimolar amounts of lactic and acetic acid (Suskovic *et al.,* 2010). Most of heterofermentative species have flavoprotein oxidases, which catalyse the reduction of oxygen, resulting in the accumulation of hydrogen peroxide. During heterofermentations, products such as formic acid, acetoin, acetaldehyde and diacetyl, which possess antimicrobial activity, can be accumulated. Malic, lactic and citric acid can be further metabolised to other antimicrobial products such as acetic acid, formic acid and CO_2 (Ogunbanwo *et al.,* 2003).

2.6.6.1 Organic Acids

The most important and best characterized antimicrobials produced by LAB are lactic and acetic acid (De Vuyst and Leroy, 2007). The amount and type of acids produced during fermentation influence the subsequent microbial activity in the fermented material. Acetic acid, for example, is more antagonistic against yeasts compared to lactic acid. Some oxidative yeasts are able to utilize organic acids as a carbon and energy source and consequently cause spoilage through deacidification in fermented, especially plant material where they are naturally present. The inhibitory effect of organic acids is mainly caused by undissociated form of the molecule, which diffuses across the cell membrane towards the more alkaline cytosol and interferes with essential metabolic functions (Suskovic *et al.,* 2010). The toxic effects of lactic and acetic acid include the reduction of intracellular pH and dissipation of the membrane potential.

2.6.6.1.1 Lactic Acid

The major metabolite of lactic acid bacteria is lactic acid, which is responsible for the significant pH changes in their growth environment – sufficient to antagonize many microorganisms (Eklund, 1989). The undissociated form of the weak organic acid diffuses over the cell membrane and is, depending on intracellular pH, more or less dissociated inside the cell, releasing H+ -ions that

acidify the cytoplasm (Axelsson, 1990; Piard and Desmazeaud, 1991). In addition to the pH effect, the undissociated form of the molecule mediates the antimicrobial effect of collapsing the electrochemical proton gradient causing bacteriostasis and eventual death of the susceptible bacteria (Eklund, 1989). The effect is more pronounced at pH values below the pKa value of the acid, i.e. when the acid is in undissociated state (Axelsson, 1990; Piard and Desmazeaud, 1991).

Reiss (1976) observed that 0.75% lactic acid (approx. 80 mM) reduced the growth of Aspergillus parasiticus (Reiss, 1976), while El-Gazzar, Rusul and Marth, (1987) found that lactic acid up to concentrations of 2% (200 mM) supported the growth of Aspergillus parasiticus (El-Gazzar, Rusul and Marth, 1987). This difference could be due either to different tolerance to organics acids between strains or to variations in their experimental designs.

2.6.6.1.2 Acetic Acid and Propionic Acid

Heterofermentative LAB produces acetic acid in presence of external electron acceptors in relatively high amounts, whereas propionic acid is only produced in trace amounts. Both acids have higher pKa values than lactic acid and therefore have a higher proportion of undissociated acid at a certain pH. Similar to lactic acid, acetic and propionic acid interact with cell membranes to neutralise the electrochemical proton gradient, but the effect of acetic and propionic acid is often dependent on the decrease in pH caused by lactic acid (Freese *et al.*, 1973; Eklund, 1989).

Propionic acid negatively influence fungal growth, especially at lower pH (Woolford, 1984), and affect fungal membranes at pH values below 4.5 (Hunter and Segel, 1973). Propionic and acetic acid also inhibit amino acid uptake (Freese *et al*., 1973; Eklund, 1989). Some salts of propionic acid, such as sodium propionate and ammonium propionate show a similar effect against yeast and filamentous moulds at low pH (Woolford, 1984). Moon (1983) found that mixtures of high concentrations of lactic, acetic and propionic acid inhibited yeast species that normally grow well

in relatively high concentrations (100 mM) of the individual acids, except for propionic acid (Moon, 1983). The combination of lactic acid produced during LAB growth and the sodium acetate of de Man, Rogosa, Sharpe (MRS) substrate (De Man *et al.*, 1960) a standard growth medium for LAB, has synergistic antifungal effects. (Cabo *et al.*, 2002; Stiles *et al.*, 2002). The sodium acetate in MRS might also have synergistic effects with additional antifungal compounds produced by LAB (Stiles *et al.*, 2002).

2.6.6.2 Other End Products

2.6.6.2.1 Hydrogen Peroxide

Most LAB possess flavoprotein oxidases (and NADH peroxidases), which enables them to produce hydrogen peroxide (H_2O_2) in the presence of oxygen. Hydrogen peroxide accumulates in the environment since LAB not produces catalase (Condon, 1987). The antimicrobial effect of hydrogen peroxide is well documented (Davidsson *et al.*, 1983) and attributed to a strong oxidizing effect on the bacterial cell, and to the destruction of basic molecular structures of cellular proteins.

The antimicrobial effect of hydrogen peroxide, even though the concentration itself is not inhibitory, may be potentiated by the presence of lactoperoxidase and thiocyanate in natural environments such as milk and saliva (Condon, 1987). The lactoperoxidase-thiocyanate-peroxide system involves the reaction of hydrogen peroxide and thiocyanate through the catalysis by lacto-peroxidase. The intermediary products such as hypothiocyanate act inhibitory to other microorganisms. Lacto-peroxidase and thiocyanate are present in milk, and when some LAB are grown in milk or milk products, the third needed component, hydrogen peroxide, is added (Björck *et al.*, 1975; Bjorck, 1978).

2.6.6.2.2 Diacetyl (2,3-Butanedione)

Diacetyl is the molecule responsible for the characteristic aroma associated with butter. It is produced by strains of all genera of lactic acid bacteria during citrate fermentation (Earnshaw, 1992). The antimicrobial effect of diacetyl is well documented, especially at pH below 7.0 (Jay, 1982). However, the amount of diacetyl needed to exert antimicrobial activity (≤ 200 mM) will dramatically alter the taste and aroma of the product (Piard and Desmazeaud, 1991).

2.6.7.2.3 Carbon dioxide

The influence of carbon dioxide on product preservation is twofold. Except for its own antimicrobial activity, it creates an anaerobic environment by replacing the existent molecular oxygen. The antifungal activity of CO_2 is due to the inhibition of enzymatic decarboxylations and to its accumulation in the membrane lipid bilayer resulting in dysfunction in permeability (Lindgren and Dobrogosz, 1990).

2.6.6.2.4 Reuterin and Reutericyclin

Selected isolates of Lactobacillus reuteri produce two compounds, reuterin and reutericyclin, both active towards Gram-positive bacteria. Reutericyclin is a tetramic acid derivative and reuterin is a mixture of monomeric, hydrated monomeric and cyclic dimeric forms of b-hydroxypropionaldehyde with a broader spectrum of inhibitory activity, including Gram-negative bacteria, fungi and protozoa (Kuleasan and Cakmakci, 2002; Gänzle and Vogel, 2003; Leroy *et al.*, 2006).

2.6.6.2.5 Other Low Molecular Mass Antimicrobials

Other low molecular mass compounds with antimicrobial activity against Gram-positive and Gram-negative bacteria, moulds and yeasts have been described, including antifungal cyclic dipeptides, phenyllactic acid, 4-hydroxyphenyllactic acid and 3-hydroxy fatty acids (Strom *et al.*,

2002; Sjogren *et al.*, 2003; Valerio *et al.*, 2004). Niku-Paavola and colleagues discovered new types of antimicrobial compounds produced by Lactobacillus plantarum (benzoic acid, methylhydantoin and mevalonolactone) active against fungi and some Gram-negative bacteria (Niku-Paavola *et al.*, 1999).

2.6.6.3 Bacteriocins of Lactic Acid Bacteria

Some of LAB produce bacteriocins, antibacterial proteinaceous substances with bactericidal activity against related species (narrow spectrum) or across genera (broad spectrum of activity) (Ogunshe *et al.,* 2007). Bacteriocin biosynthesis is a desirable characteristic for strain selection as it serves as an important mechanism of pathogen exclusion in fermented foods as well as in the gastrointestinal environment. Bacteriocins are ribosomally synthesized peptides or proteins with antimicrobial activity produced by many Gram-positive and Gram-negative bacteria; however, those produced by food grade LAB have received considerable attention due to their potential application in food industry as natural preservatives (biopreservatives). LAB bacteriocins are small antimicrobial peptides or proteins that possess activity towards closely related Gram-positive bacteria, whereas producer cells are immune to their own bacteriocins (Klaenhammer, 1988; Chen and Hoover, 2003). There are several proposed bacteriocin classifications divided into 3 or 4 classes:

(i) lantibiotics or small, heat-stable, lanthionine-containing, single- and two-peptide bacteriocins (class I), whose biologically inactive prepeptides are subjected to extensive post-translational modification;

(ii) small, heat-stable, non- -lanthionine-containing bacteriocins (class II), including pediocins like or Listeria-active bacteriocins (class IIa), two-peptide bacteriocins (class IIb) and circular bacteriocins (class IIc);

(iii) bacteriolysins or large, heat-labile, lytic proteins, often murein hydrolases (class III) (Klaenhammer, 1988; De Vuyst and Leroy, 2007). Some authors (Klaenhammer, 1993; Nes *et al.*, 1996) also proposed

(iv) class IV bacteriocins that require non-proteinaceous moieties (lipid, carbohydrate) for their activity (Klaenhammer, 1993; Cotter *et al.*, 2005; De Vuyst and Leroy, 2007).

Lantibiotics are small (30 kDa), heat-labile antimicrobial proteins not as well characterised, whose mechanism of action is distinct in function as they lyse the sensitive cells by catalysing cell-wall hydrolysis (Cotter *et al*., 2005). Only four LAB bacteriolysins have been genetically characterised so far (Joerger and Klaenhammer, 1990; Nilsen *et al.*, 2003), although the non-LAB bacteriolysins have been identified.

Class IV of complex bacteriocins that require non-proteinaceous moieties like carbohydrate or lipid for their activity has also been suggested by some authors (Klaenhammer, 1993); however, bacteriocins in this class have not been characterised convincingly, hence definition of this class requires additional characterization (Chen and Hoover, 2003; Cotter *et al.*, 2005).

2.6.6.4 Mode of Bacteriocin Action

Bacteriocins that are produced by LAB can be of broad or narrow spectrum, but in general, the activity is directed against low G+C Gram-positive species (Cotter *et al.*, 2005). The antibacterial spectrum includes spoilage organisms and foodborne pathogens such as *Listeria monocytogenes* and *Staphylococcus aureus*. Wide ranges of mode of action have been described for bacteriocins, such as enzyme activity modulation, inhibition of outgrowth of spores and formation of pores in cell membrane. Most bacteriocins interact with anionic lipids that are abundantly present in the membranes, and consequently initiate the formation of pores in the membranes of susceptible cells (Chen and Hoover, 2003). However, membrane disruption models cannot adequately describe the

mode of action of bacteriocins. Rather, specific targets seem to be involved in pore formation and other activities. For the nisin and epidermin family of lantibiotics, the membrane-bound cell wall precursor lipid II has been identified as target (Héchard and Sahl, 2002). Most of class II bacteriocins dissipate the proton motive force (PMF) of the target cell via pore formation (Venema *et al.*, 1995). The subclass IIa bacteriocin activity depends on a mannose permease of the phosphotransferase system (PTS) as a specific target. The subclass IIb bacteriocins (two-component) also induce dissipation of the PMF by forming cation- or anion-specific pores; specific targets have not yet been identified. Finally, subclass IIc comprises miscellaneous peptides with various modes of action such as membrane permeabilisation, specific inhibition of septum formation and pheromone activity (Cotter *et al.*, 2005).

2.6.6.5 Resistance and Immunity to Bacteriocins

Bacteriocin producer has developed protection mechanisms against its own bacteriocin. Two distinct systems of bacteriocin immunity in the producing cell have been identified. Protection can be mediated by dedicated immunity protein and/or a specialised ABC-transporter system involving two or three subunits that probably pump the bacteriocin through the producer membrane. These two immunity systems can work synergistically to protect the producing cells from their own bacteriocin (Klein and Entian, 1994). In the case of lantibiotic immunity, e.g. protein LanI, which is most likely localised at the cytoplasmic membrane, probably confers immunity to the producer cell by preventing pore formation by the bacteriocin. Related ABC-transporter system LanEFG probably acts by excreting bacteriocins that were inserted into the membrane back to the extracellular microenvironment and thus keeping bacteriocin concentration in the membrane under a critical level (De Vuyst and Leroy, 2007). Regulation of bacteriocin production and immunity is

most frequently mediated through two-component signal-transduction systems, often as part of the quorum-sensing mechanism (Quadri, 2002).

2.6.6.6 Use of Bacteriocins in Combination with Other Antimicrobial Factors

The antimicrobial spectra and activity of bacteriocins can be extended through the synergy between different antimicrobial factors such as inorganic salts (especially sodium chloride), organic acids and their salts, chelating agents (such as EDTA), essential oils and their active components, phenolic compounds, as well as other natural antimicrobials (Deegan *et al.*, 2006).

Application of bacteriocins together with different physicochemical treatments, like heat treatment, modified atmosphere packaging, high hydrostatic pressure, pulsed electric field, pulsed magnetic field and gamma irradiation, has received great attention in recent years (Chen and Hoover, 2003; Ross *et al*., 2003; Galvez *et al.*, 2007). The effectiveness of bacteriocins in combination with hurdle technology will depend on the type of food and its natural microflora.

Thus with acidification of the food acidotolerant bacteria may be selected, while heat treatment may favour bacterial endospores, but in combination with bacteriocins higher sensitization may be achieved after optimization of doses and conditions. Furthermore, the Gram-negative bacteria could become sensitive to bacteriocin activity upon exposure to hurdles such as chelating agents that destabilize the bacterial outer membrane (Fang and Tsai, 2003; Omar *et al.*, 2006; Galvez *et al.*, 2007).

2.6.6.6 Application Bacteriocin in Food Industry

Nisin is the foremost bacteriocin licensed as food preservative (E234). Commercial production of nisin by Lactococcus lactis ssp. lactis began in England in 1953, and international acceptance of nisin was given in 1969 by the Joint Food and Agriculture Organisation/World Health

Organization (FAO/WHO) (Joint FAO/WHO, 2002). In 1988, it was approved by the US Food and Drug Administration (US FDA) for use in pasteurized, processed cheese spreads and since then, as a food additive in over 50 countries (Cotter *et al.*, 2005). Nowadays, the most established available form of nisin for use as a food preservative is Nisaplin^{TM}. Applications of nisin have been developed for processed cheese, dairy desserts, milk, fermented beverages, bacon, frankfurters and fish, often in combination with hurdle technologies to achieve better inhibitory effect (Vanderbergh, 1993; Guinane *et al*., 2005; de Arauz *et al*., 2009). Its use extends shelf life of the food by inhibition of Gram-positive spoilage bacteria such as Listeria, Staphylococcus and Mycobacterium, and spore-forming bacteria Bacillus and Clostridium (Olasupo *et al*., 2004; Stergiou *et al*., 2006). The spores of these bacteria are more sensitive to nisin than their vegetative cells, so nisin is often applied in heat-processed food such as canned vegetables. The spectrum of its activity can be successfully broadened when it is applied in combination with chelating agent such as EDTA (Bari *et al*., 2005). Very few variants of six naturally occurring nisin molecules are described with enhanced activity against Gram-positive pathogens (Field *et al*., 2008).

2.6.6.7 The Role of Antimicrobial Activity of Probiotic Lactic Acid Bacteria in Prevention and Treatment of Infections

The widespread use of antibiotics in treatment of infections resulted in increased number of antibiotic resistant bacteria, fewer treatment options and most antibiotics ineffective. Alternative antimicrobial strategies in the treatment and prevention of gastrointestinal infections are the application of probiotics and their antimicrobial metabolites such as bacteriocins. Probiotic is a mono- or mixed culture of live microorganisms which, applied to animal or man, affect beneficially the host by improving the properties of the indigenous microflora, according to broader Fuller's definition (Fuller, 1989), proposed by Havenaar and Huis in't Veld (Periti and

Tonelli, 2002). Probiotics are largely administered through functional foods and as dietary supplements (pharmaceuticals) or biotherapeutics (approved drugs with important therapeutic applications) (Periti and Tonelli, 2002). Lactic acid bacteria are the most important probiotic microorganisms because they are autochthonous in the human gastrointestinal tract of healthy people (Goldin and Gorbach, 1992; Tannock, 1999). A considerable number of health benefits have been postulated as a result of the probiotic intake, including modification of gut microflora, prevention of pathogen colonisation, stimulation of gut immunity, reduction in inflammatory reactions, prevention of colon cancer, alleviation of lactose intolerance, lowering of serum cholesterol and reduction of food allergies. Each property is strain-dependent, and must be confirmed by in vitro experiments, animal experiments and clinical trials. Mode of action of probiotics includes antagonistic effects against pathogenic microorganisms in intestinal tract (embracing multiple mechanisms for preventing infection), alteration of microbial metabolism in the intestinal tract, stimulation of immunity and increase of nutritional value of food. Much of the benefit derived from probiotic LAB is a consequence of their ability to acidify the intestine by producing the lactic acid and thus create a hostile environment for pathogens. Besides lactic acid, probiotic bacteria can also produce antimicrobials such as hydrogen peroxide, bacteriocins, short-chain fatty acids such as acetic, propionic and butyric acid, rendering vital nutrients unavailable to pathogens and altering the redox potential of the intestinal environment. There is also considerable evidence that deconjugation of conjugated bile salts in the intestine is the mechanism of the resistance of probiotic bacteria to high concentration of bile salts in small intestine, which are inhibitory for Gram-positive bacteria, but have little effect against Gram-negative bacteria.

However, upon deconjugation, the free bile acids are more toxic for both, Gram-positive and Gram-negative microorganisms, which is also one of the antagonistic mechanisms against

pathogens in the intestine (Kos, 2001; Floch, 2002). Another line of probiotic defence against infection in the intestinal tract is the enhancement of intestinal barrier function by the promotion of mucin production and by colonisation resistance mechanism, which prevents colonisation of the intestine by pathogens (Saulnier *et al.*, 2009). Colonization resistance is apparent in two major regions of the intestinal habitat: the luminal contents and the mucosal surfaces. Adherence factors on the surface of probiotic cells, mostly proteins or polysaccharides, may promote pathogen exclusion, mucosal integrity and host immunomodulation. Comparative genome analyses confirmed the role of mucus-binding proteins (MUB) in intestinal mucus adherence of Lactobacillus strains isolated from the intestine. Namely, the MUB domains found exclusively in intestinal lactobacilli suggest that these proteins mediate specific interactions or functions between these microbes and their hosts (Demeria *et al.*, 2009; O'Flaherty *et al.*, 2009). Cell surface structures such as teichoic acids, lipoteichoic acids and surface layer proteins (S-layers) have also been reported as important for probiotic adhesion and immunomodulation. S-layer proteins from different strains of L. acidophilus, L. helveticus, L. brevis, L. kefir and L. crispatus have been shown to be involved in mediating adhesion to different host surfaces (Avall-Jaaskelainen and Palva, 2005; de Leeuw *et al.*, 2006). Additionally, some of them are found to prevent adhesion of the foodborne pathogens, such as Escherichia coli and Salmonella enterica serovar Typhimurium, to cultured intestinal epithelial cell lines, to frozen sections of intestinal tissue, as well as to intestinal mucus and uroepithelial cells (Kos, 2001; Golowczyc *et al.*, 2007; Antikainen *et al.*, 2009). Surface proteins have also been characterised as key factors involved in immunomodulation (Valeur *et al.*, 2004; Beganovi, 2008). Not only LAB themselves were reported to activate immune cells and to confer enhanced protection against enteropathogens (Shu *et al.*, 2000; Perdigon *et al.*, 2004; Omar *et al.*, 2006). Non-bacterial fractions of fermented milk, containing bacterial metabolites produced during fermentation by LAB, were effective in induction of different

cytokine patterns and enhanced protection against enteropathogens in mice (De Moreno de LeBlanc *et al*., 2005; Vinderola *et al.*, 2007). The mechanisms behind the prevention of gastrointestinal and urinary tract infections by probiotic bacteria have been elucidated in animal, but also in human studies, confirming enhancement of immune responses and production of antimicrobial substances (Vanderbergh, 1993; Weizman *et al.*, 2005; Reid and Bruce, 2006; Antikainen *et al.*, 2009). However, there is increasing clinical evidence that probiotics are effective not only in the treatment and prevention of gastrointestinal diseases, but also in chronic liver disease, multiple organ dysfunction syndrome and autoimmune disease (Loguercio *et al*., 2005; Alberda *et al.*, 2007; Matsuzaki *et al.*, 2007; Demeria *et al.*, 2009).

2.6.7 Bacteriocin-Producing Starter and Non-Starter Lactic Acid Bacteria in Food

Industry, besides the well-known biopreservative effects of antimicrobial metabolites of lactic acid bacteria such as lactic acid, acetic acid, hydrogen peroxide and diacetyl, bacteriocins have the most immediate potential in food application as biopreservatives and they can be readily introduced into food without any concentration or purification (Cotter *et al.*, 2005). Since lactic acid bacteria are generally regarded as safe (GRAS) according to the FDA, they could be used in food production and food biopreservation.

2.6.7.1 Bacteriocin-producing Starter Cultures

The main antimicrobial effect of starter LAB, responsible for biopreservation, is the rate of acidification, but in slightly acidified products or to eliminate undesirable microorganisms that display acid tolerance, such as Listeria monocytogenes, the bacteriocinogenic activity could play a crucial role. The use of bacteriocin-producing starter cultures may not only contribute to food safety, but also prevent the growth of undesirable autochthonous lactic acid bacteria that produce off-flavour. This property may improve the competitiveness of the starter cultures and lead to a

more controlled and standardized fermentation process as it has been shown in sourdough, fermented sausage, fermented vegetables and olives, and cheese production (Ross *et al.*, 2000; De Vuyst *et al.*, 2004; Leroy *et al.*, 2006).

2.6.7.2 Bacteriocin-producing Adjunct Cultures

Bacteriocin producers can be delivered to a food product as an adjunct culture, together with the starter culture. In this case, the ability of starter adjunct to grow and produce bacteriocin in the product is crucial for its successful use. The bacteriocin-producing adjunct cultures are mostly isolated from raw milk, vegetables, cereals and other natural sources of lactic acid bacteria that are believed to contain strains essential not only for the characteristic flavour of traditional fermented products, but also with promising and useful properties such as bacteriocinogenic activity, which will make them applicable as starters. For example, Lactococcus lactis strain, which produces both nisin and lacticin 481, isolated from raw ewe's milk, might be used as adjunct culture to the commercial starter in the manufacture of dairy products to inhibit or destroy undesired microorganisms (Bravo *et al.*, 2009). Adjunct culture does not need to contribute to the flavour but it is important that the starter culture is resistant to bacteriocin produced by the adjunct culture.

One of the exceptions is the controlled lysis of starter culture during cheese manufacture caused by bacteriocin-producing strain, with the aim to release intracellular enzymes, needed for accelerated ripening and improvement of product flavour (O'Sullivan *et al.*, 2003; Cotter *et al.*, 2005).

2.6.7.3 Bacteriocin-Producing Protective Cultures

Bacteriocinogenic protective cultures alone can be used to inhibit spoilage and pathogenic bacteria during the shelf life of non-fermented foods by producing bacteriocin in situ or previously cultured in growth medium and after that applied as an ingredient in food processing. Two preparations are

already present on the market: ALTATM 2341, containing pediocin PA1 produced by Pediococcus acidilactici, and Microgard™, a commercially available fermented milk product containing antimicrobial metabolites. In the literature different milk-based preparations such as lacticin 3147 are described (Guinane *et al.*, 2005). The addition of purified or semi-purified bacteriocins as food preservatives requires approval from legislative point of view. There is also a problem of costly production because of low production rates, instability and expensive downstream processing of bacteriocins. If immobilized or microencapsulated bacteriocin or bacteriocinogenic strain is applied on the food surface, much lower concentration is needed compared to the application in the whole food volume (Champagne and Fustier, 2007; Galvez *et al.*, 2007). Other advantages of immobilized bacteriocins are the possibility of gradient-dependent, continuous supply of bacteriocin and the protection against food components and enzymatic inactivation. The use of antimicrobial films containing immobilized bacteriocins for the development of antimicrobial packaging is a recently developed technique (La Storia *et al.*, 2008; Papagianni and Anastasiadou, 2009)

2.7 GUT MICROFLORA

The human colon is the body's most metabolically active organ (Glenn and Roberfroid, 1995). This is because of the resident microbiota, which comprises of over 1,000 bacterial cells for every gram of gut contents (Glenn and Roberfroid, 1995; Macfarlane and Macfarlane, 2012).

In terms of the microbiology of different digestive tract areas, there is variability both in terms of composition and activity (Saulnier *et al.*, 2009). The lumen of the human stomach is essentially sterile due to a low gastric pH (Desai, 2008). However, micro-organisms are known to reside in the mucosal layer that overlies the gastric epithelium. This includes *Helicobacter pylori*, which has attracted a great deal of research interest (Gibson and McCartney, 1998; Jama, 2013). This

organism uses its flagellae to invade the gastric mucus layer and thereafter adhere to epithelial cells (Jama, 2013). In conjunction with a production of ammonia, this allows effective colonization of the stomach (Rathbone and Heatley, 1992). In the small intestine, the transit time of gut contents tends to maintain bacterial numbers at below 106/ml of contents (Gibson and McCartney, 1998).

Facultatively anaerobic and aerotolerant bacteria such as streptococci, staphylococci and lactobacilli dominate the upper small gut with bacterial numbers showing a progressive increase (Gibson *et al.*, 1996). In comparison to other regions of the gastrointestinal tract, the human large intestine is an extremely complex microbial ecosystem, with at least several hundred different bacterial species being present (Glenn and Roberfroid, 1995). The environment is favourable for bacterial growth with a slow transit time, ready availability of nutrients and favourable pH (Gibson *et al.*, 2010). The vast majority (>90%) of the total cells in the body are present as bacteria in the colon. It is thought that over 60% of the faecal mass exists as prokaryotic cells. Generally, the various components of the large intestinal microbiota may be considered as exerting pathogenic effects or they may have potential health promoting values (Gibson *et al.*, 1996; Gibson *et al.*, 2010). Given that the microbiota has components that are positive for human health, there is much interest in the use of diet to specifically increase groups perceived as health promoting (Manning and Gibson, 2004). As such, the gastrointestinal flora and its activities are a major focus for functional food developments.

2.7.1 IMPORTANCE OF AN INDIGENOUS MICROFLORA

A well-established and matured indigenous microflora on external and internal body surfaces of animals and man is very stable. The penetration and colonisation of non-indigenous microorganisms from the environment and/or from other animal species (xenochthonous microorganisms) onto these body surfaces is hindered (Desai, 2008). The importance of an

indigenous microflora in the gut as a natural resistance factor against potential pathogenic microorganisms was already recognised in the 19th century by Metchnikoff during his research on cholera (Podolsky, 1998). Many decades later the role of the indigenous microflora received renewed interest after findings in laboratory animals orally treated with antibiotics. The antibiotics caused intestinal disturbances owing to infectious agents. It was suggested that this effect was induced by suppressing the normal gut microflora (Havenaar and Huis, 1992). Later on, the protective effect of the normal intestinal microflora in chickens against *Salmonella infantis* infection (Nurmi and Rantala, 1973). Colonisation resistance of the gut microflora was further confirmed for Salmonella and for other pathogenic bacteria such as *Escherichia coli*, *Clostridium* and *Yersinia enterocolitica*. There has been demonstrated in 'normalisation' studies with germ-free animals inoculated with several dilutions of the total intestinal microflora from normal animals of the same species (Impey *et al.*, 1982). The colonisation resistance induced by an indigenous microflora is partly based on occupation of available niches (competitive inhibition of binding sites) and autogenic regulation factors (e.g. synthesis of fatty acids, hydrogen peroxide, bacteriocins). Another important factor might be the non-specific activation of the immune system (Impey *et al.*, 1982). The gut, the mucosa, as well as the skin, have humoral and cellular immune systems which can influence the composition of the microflora (gut microflora). It has been shown that the activity of the immune system of germfree animals is very low since less γ-globulin, smaller lymph nodes and fewer lymphocytes and phagocytes were found. Activation of macrophages was noticed following the introduction of indigenous microorganisms. In addition to this stimulating effect on non-specific resistance factors, the gut microflora has also an important complimentary function in the digestion of dietary components, such as plant polymers and the synthesis of vitamins (Wood, 1996).

2.7.2 GASTROINTESTINAL BACTERIA INFLUENCED BY DIET

Most work on the microbes of the large bowel of adults has concentrated on the analysis of fecal samples. The composition of the fecal microbiota has been the subject of many investigations and has been summarized in numerous reviews. Most studies focus on enumeration of the major groups of microbes with some studies characterised to the genus level. Few workers have been as thorough as Moore and Holdeman (1974) who identified the species level wherever possible. Populations of faecal bacteria constitute a major proportion (approximately 50%) of feces (Tomomatsu, 1994).

2.7.3 STRUCTURE AND FUNCTION OF THE GASTROINTESTINAL TRACT (GI)

The gastrointestinal tract is a tube extending from the lips to the anus and is divided into various well-defined anatomical regions. The digestive and absorptive functions are well known but, in addition to being an organ in the body, the intestine acts as a container for the most intimate portion of the chemical environment. Assimilation of food is not the only physiological function of the alimentary tract. It is also concerned with the excretion of chemical waste, the control of body metabolism and immune response (Fooks *et al.*, 1999). Furthermore, the gut harbours a complex ecosystem.

2.7.4 MICROBIOLOGICAL ASPECTS OF LARGE INTESTINE

The large intestine harbours the largest and most complex microbial ecosystem associated with the human body, consisting of several hundred different strains of anaerobic bacteria, with numbers exceeding 1011/g of intestinal contents (Fooks *et al.*, 1999; McBain and Macfarlane, 1998). This is because of the resident microbiota, which comprises 1012 bacterial cells for every gram of gut contents. As the large intestine usually contains about 200 g of contents, there is enormous biological activity. The fact that these activities can be modulated or perhaps even controlled through diet is of high relevance (Gibson *et al.*, 2000). The microbiota is involved in the catabolism

of a vast range of dietary and endogenously secreted compounds. The products of these biotransformations are often toxicological significance to the host. For example, the occurrence of colon cancer is greatly influenced by diet, while metabolism of dietary components by intestinal bacteria has been demonstrated to be an important factor in tumour initiation. The colonic microflora may be involved in the aetiology of large bowel cancer by chemical modification or activation of a wide variety of chemical agents with carcinogenic or cocarcinogenic potential.

Exposure of the intestinal microbiota to potential toxicants may occur due to their presence in the diet by biliary excretion of endogenously metabolised substances into the intestine, enzymic activation of procarcinogens by the gut microflora or by direct production ofmutagenic substances by intestinal microorganisms (McBain and Macfarlane, 1998). In terms of functionality, the human colon is the body's most metabolically active organ. Besides vertical transmission of microorganisms, the body surfaces mentioned are contaminated during and directly after birth with a variety of microbial strains from the immediate environment (horizontal transmission) (Havenaar and Huis, 1992; Doyle and Buchanan, 2012). A number of these microorganisms will colonise these body surfaces permanently or temporarily, while others disappear. The temporarily colonising strains act as pioneers, which initiate the successive domiciliation of other microorganisms (Havenaar and Huis, 1992; Desai, 2008). During time, under normal conditions, the microflora will mature to a balanced composition of many different microorganisms. The occupation of special niches depends on the local environmental circumstances in these specific habitats. These local conditions are determined by multifactorial interactive processes between the host and the microorganisms (Havenaar and Huis, 1992; Desai, 2008). Therefore, the mature composition of the natural microflora is specific to the animal species and even specific to an individual (Havenaar and Huis, 1992). In general, the natural microflora is called the indigenous

microflora of a given species (including autochthonous and allochthonous microorganisms), and the indigenous microflora of a given individual (mainly autochthonous microorganisms), respectively. However, the terminology around symbiosis of microorganisms on body surfaces is often confusing. Species specificity of the microflora has been shown in several studies (Havenaar and Huis, 1992).

2.7.5 BACTERIAL FERMENTATIONS IN THE LARGE INTESTINE

It is clear that a complex, resident gut flora is present in humans. While the transit of residual foodstuffs through the stomach and small intestine is probably too rapid for the microflora to exert a significant impact, this slows markedly in the colon (Roberfroid *et al.*, 2010). The average transit time is around 70 hours, but can be higher. As such, colonic micro-organisms have ample opportunity to degrade available substrates (Desai, 2008). These may be derived from either the diet or by endogenous secretions (Desai, 2008). Fermentations by gut bacteria consist of a series of energy yielding reactions that do not use oxygen in the respiratory chains. The electron acceptors may be organic (e.g. some products of the fermentations) or inorganic (e.g. sulphate, nitrate) (Desai, 2008; Gibson *et al.*, 2010). As carbohydrates form the principal precursors for fermentation, ATP is usually formed through substrate level phosphorylation by saccharolytic micro-organisms (Macfarlane and Gibson, 1997). The fermentation process in the large gut is influenced by a variety of physical, chemical, biological and environmental factors.

Major substrates available for the colonic fermentation are starches that, for various reasons, are resistant to the actions of pancreatic amylases and can be degraded by bacterial enzymes as well as dietary fibers like pectins and xylans (Desai, 2008). Other carbohydrate sources available for fermentation in lower concentrations include oligosaccharides and a variety of sugars and non-absorbable sugar alcohols (Gibson *et al.*, 1996).

2.7.6 METABOLITES PRODUCED IN THE LARGE INTESTINE

In terms of end products, a variety of different metabolites arise. Predominant among these are the short chain fatty acids (SCFA), acetate, propionate and butyrate (Cummings *et al.*, 1987). The majority areabsorbed into the bloodstream and can be further metabolised systemically. Transport to, and further metabolism of SCFA in the liver, muscle or other peripheral tissues is thought to contribute about 7 to 8% of host daily energy requirements (Gibson *et al.*, 2010). Other products include metabolites, such as ethanol, pyruvate and lactate, which are mostly further converted to SCFA and therefore not allowed to accumulate to any significant level in the large bowel (Desai, 2008; Gibson *et al.*, 2010).

2.8 DIARRHOEA

Diarrhoea is a symptom marked by rapid and frequent passage of semisolid or liquid fecal material through the gastrointestinal tract and involves both an increase in the motility of the gastrointestinal tract along with increased secretions and a decrease in the absorption of fluid and thus loss of electrolytes particularly Na+ and water (Rang *et al*., 2003). It is one of the most common clinical signs of gastrointestinal disease, but also can reflect primary disorders outside the digestive system. Diarrhoea is considered to be present if one of the following conditions are met:

(a) stool weight of greater than 200 g per day;

(b) more than 2 stools per day for more than 30 days;

(c) more than 3 stools per day for more than 7 days;

(d) more than 3 stools per day, looser than usual, for more than 3 days; or

(e) more than 3 stools per day, with a change in frequency or consistency (Sarin, 2012).

2.8.1 CLASSIFICATION OF DIARRHOEA

Based on time course, diarrhoea may be classified as acute, persistent, and chronic.

Acute diarrhoea is defined as three or more loose bowel movements in a 24-hour period (Gregorio *et al*., 2009) and the duration is less than 2 weeks (Halsey, 2009). Diarrhoea is said to be persistent if the duration varies from 2 to 4 weeks, and chronic if it lasts more than 4 weeks in duration (Guerrant *et al*., 2001). It can also be classified based on the etiology as infectious or non-infectious. Non-infectious diarrhoea, e.g. due to irritable bowel syndrome, results from a complex interaction of immune and neuronal factors. The mechanisms of diarrhoea caused by various pathogens can be classified as inflammatory or non-inflammatory (De Hostos *et al*., 2011). Diarrhoea can also be classified based on its pathophysiology into secretory, osmotic, inflammatory, iatrogenic/drug related, and functional/motility-related diarrhoea (Guandalini and Vaziri, 2011).

2.8.1.1 Secretory Diarrhoea

The basic pathophysiology involves either net secretion of ions (chloride or bicarbonate) or inhibition of net sodium absorption (Poley and Hofmann, 1976; Hoque *et al.,* 2012).

Most causes of secretory diarrhoea alter the second messenger systems through alteration in cAMP, cGMP, or intracellular calcium-regulated ion transport pathways (Field, 2003; Binder, 2005; Hoque *et al.,* 2012) Alterations in these mediators cause the cystic fibrosis transmembrane conductance regulator (CFTR) mediated Clsecretion and inhibition of small intestinal-coupled Na+–Cl- transport (Salazar-Lindo, 2011; Hoque *et al.*, 2012). CFTR is a chloride channel and is the primary driver of secretion in cases of diarrhoea caused by enterotoxigenic bacteria (De Hostos *et al*., 2011). Secretory diarrhoea may arise from infectious and non-infectious causes. The most common cause of secretory diarrhoea is infection, which usually affect the small intestine (Schiller,

1999). Infectious secretagogues include the viruses: rotavirus and norovirus, enterotoxigenic *E. coli* (ETEC), *Vibrio cholerae*, *Giardia*, and *Cryptosporidium* infections (Hoque *et al*., 2012). Non-infectious secretagogues include chemicals produced by certain types of cancer, prostaglandins produced in patients with bowel inflammation and substances not well absorbed such as fatty acids and bile acid (Bliss *et al*., 2006). Secretory diarrhoea persists in spite of fasting (Mercadante, 1995).

2.8.1.2 Osmotic Diarrhoea

Osmotic diarrhoea occurs when osmotically active but poorly absorbable solutes in the intestine draw water to them because the gastrointestinal mucosa is not able to maintain an osmotic gradient (Sellin, 2001). The causes of osmotic diarrhoea are varied but can be broken down into decreased enzymatic availability (lactose intolerance), a genetic abnormality that decreases or eliminates the ability of the body to absorb certain nutrients (celiac sprue), poorly absorbable sugars (sorbitol, mannitol or lactose) (Strasinger and Di Lorenzo, 2008), and poorly absorbable solutes (magnesium, sulfates, and phosphates) (Hammer *et al.*, 1989). This fecal matter then creates a negative osmotic gradient causing leakage of more fluid into the gut increasing the stool volume and resulting in diarrhoea (Field, 2003). Osmotic diarrhoea typically lessens after fasting or stopping the causative agents (Mercadante, 1995).

2.8.1.3 Inflammatory Diarrhoea

Inflammatory diarrhoea may result from a wide variety of etiologies including infections and Inflammatory Bowel Diseases (IBDs). Infectious pathogens causinginflammatory diarrhoea primarily affect the distal small bowel or the colon (Pawlowski *et al.*, 2009). They cause disease either by elaborating cytotoxins or by invading the epithelium with resultant recruitment of inflammatory cells (Navaneethan and Giannella, 2008). Most of the pathogens causing

inflammatory diarrhoea do so by producing mucosal damage as well as by stimulating intestinal secretion. Agents responsible for inflammatory diarrhoea include agents such as enterotoxins, cytokines, prostaglandins, and nitric oxide (NO) that could be produced in the course of the disease (Urayama and Chang, 1997). Inflammatory bowel diseases (IBDs) are also one of the most common and important causes of inflammatory diarrhoea (Binder, 2009). The cytokines and eicosanoids initiated by inflammation down regulate the ion transporters in the colon and small bowel resulting in Na+ malabsorption (Amasheh *et al.,* 2004; Thevarajah *et al*., 2005).

2.8.1.4 Drug-Induced Diarrhoea

There are a number of drugs that are known to cause diarrhoea either as a side effect or as the desired effect of the drug. The mechanism of causing diarrhoea can vary from drug to drug. Antibiotic use may alter the bacterial flora in the colon resulting in impaired colonic salvage of malabsorbed carbohydrates (McFarland, 2006) and emergence of pathogenic organisms such as *Clostridium difficile* (Bergogne-Berezin, 2000) to cause antibiotic associated diarrhoea (AAD). Some of the drugs like lactulose may cause osmotic diarrhoea, while others may cause secretory diarrhoea. Both coffee and theophylline increase intracellular cAMP, opening of chloride channels and increasing secretion, while erythromycin interacts with the motilin receptors increasing the motility to cause diarrhoea. Prostaglandin analogs (e.g. misoprostol) can affect the intestine at many level including permeability, motility, transport of electrolytes as well as affecting peptides that stimulate secretion Similarly chemotherapeutic drugs may cause diarrhoea because of decreased rate of proliferation of the enterocytes (Sellin, 2001).

2.8.1.5 Functional Diarrhoea

When the intestines are not functioning normally, motility can be either increased or decreased and both can lead to diarrhoea. Increased motility may decrease the time for the luminal contents

to be in contact with the epithelium for absorption resulting in secretory diarrhoea, like in irritable bowel syndrome (IBS) (Prior *et al.*, 1990; Vassallo *et al.*, 1992; Drossman *et al.,* 2002) and disturbances in the neural control (Grundy, 2002; Mamikunian *et al*., 2009). On the other hand, slow transit as occurring in diabetes mellitus and scleroderma may be associated with bacterial overgrowth and the ensuing bile acid deconjugation and poor micelle formation (Camilleri, 2004).

2.8.2 EPIDEMIOLOGY OF DIARRHOEA

Although diarrhoea is a preventable disease, it remains the second leading cause of death (after pneumonia) among children aged under five years worldwide (Kaplan *et al*., 2013). Diarrhoea is one of the primary causes of morbidity and mortality on a global scale, leading to one billion disease episodes and 1.8 million deaths each year (Chou *et al*., 2010 UNICEF, 2013). It is also one of the leading causes of morbidity and mortality in developing countries among under the same age group (Regassa *et al*., 2008). It kills more young children than AIDS, malaria and measles combined (Hostos *et al.*, 2011).

Around 50% of deaths among children under five occur in sub-Saharan Africa and 40% in South Asia (Kaplan *et al.*, 2013). Such areas are also with higher case-fatality rates compared to children living in high-income countries due to lack of access to quality health care and timely and effective treatment with oral rehydration solution (ORS) and zinc (Sutariya *et al*., 2011).

Nigeria is among the five mostly poor and populous countries which account about half of the world's deaths due to pneumonia and diarrhoea, along with four other countries such as India, Ethiopia, Democratic Republic of the Congo, and Pakistan (UNICEF, 2012). Poverty, crowding, contaminated water supplies (Bakare *et al.*, 2011), childhood underweight, suboptimal breastfeeding, unsafe drinking water and sanitation, vitamin A deficiency, and zinc deficiency all contribute to its incidence (Kaplan *et al.*, 2013).

2.8.3 AETIOLOGY AND PREVENTION OF DIARRHOEA

Diarrhoeal diseases are caused by a variety of pathogens including viruses (for example, rotavirus), bacteria (*Cholera*, *Shigella* and enterotoxigenic *Escherichia coli* (ETEC)), protozoa (*Cryptosporidium* and *Entamoeba histolytica*) and helminthes. These pathogens present in the gut causing disruption of normal fluid secretion and motility and stimulating the gut to expel the contents. Most pathogens are transmitted from the stool of one person to the mouth of another via contaminated food or water (faecal-oral transmission) (Panda *et al.*, 2012; Kaplan *et al.*, 2013).

Overeating or eating of wrong foods, putrefaction of food in the intestinal tract, fermentation caused by incomplete carbohydrate digestion, nervous irritability, use of antibiotic drugs, and excessive intake of laxatives can also cause diarrhoea (Singh and Verma, 2012).

Diarrhoea also occurs frequently in post-transplantation patients who are receiving immunosuppressive drugs (Sellin, 2001). Measures for prevention of diarrhoea include: exclusive breastfeeding for the first six months of life, safe drinking water, improved sanitation, personal and food hygiene and rotavirus vaccination. The parents should also be informed about the routes of transmission of enteropathogens and preventive measures (Singh and Verma, 2012).

2.8.4 NORMAL INTESTINAL PHYSIOLOGY AND PATHOPHYSIOLOGY OF DIARRHOEA

2.8.4.1 Normal Intestinal Physiology

As diarrhoea is the end result of a derangement in the normal physiology of the intestinal handling of water and electrolyte, an understanding of these processes is essential to appreciate the pathophysiological changes that lead to diarrhoea. All segments of intestine from duodenum to distal colon have mechanisms for both absorbing and secreting water and electrolytes (Field, 2003).

There is a constant bidirectional flux of water and ions across the small intestinal mucosa, i.e., absorption and secretion. Absorption occurs in villus cells and secretion largely by crypt cells (Binder and Reuben, 2005). Sodium and water absorption by enterocytes is mediated by an active, ATP-dependent active sodium pump (Na+, K+-ATPase) located on the basolateral membranes of intestinal crypt and villus cells. In the intestine, solute movement creates the osmotic force for fluid movement. Na+ absorption drives fluid reabsorption, while active Cl- secretion contributes to water secretion in secretory diarrhoea. Small intestinal Na+ absorption is mediated primarily by two mechanisms: a glucose- or amino acid-stimulated cotransport in which Na+ accompanies the other solute and a coupled Na+–Cl- mechanism. The latter is a combination of Na+–H+ exchange and Cl-–HCO3- exchange. Short-chain fatty acid (SCFA)-mediated Na+ absorption and aldosterone-sensitive Na+ absorption occur in the colon (Binder, 2005). Among the various mechanisms described, the coupled Na+–Cl- pathways are primarily regulated by cyclic adenosine monophosphate (cAMP) levels and also by cyclic Guanosine Monophosphate (cGMP) and intracellular Ca2+ levels (Field, 2003). In addition to the transporters, there are multiple extracellular factors regulating epithelial ion transport – paracrine, immunological, neural, and endocrine factors, termed together as a single regulatory system known as PINES (paracrine–immuno-neuroendocrine system) (Mourad *et al.*, 1995).

In addition to the absorptive and secretary function of the intestine, motor functions also play a key role in facilitating digestion and absorption of fluids and nutrients. Synchronized migrating motor complexes normally occur during fasting in the stomach and small bowel with increased contractions following feeding with the total small bowel transit time of approximately 3 hours for the food reaches the colon (Kerlin *et al.*, 1982). In the colon, there is further reabsorption with the ascending and transverse colon serving as reservoirs and with the sigmoid and rectum serving as

volitional reservoirs (Proano *et al*., 1990). Any disturbance in the coordinated flux of water and ions and motility can result in the clinical syndrome of diarrhoea.

2.8.4.2 Pathophysiology of Diarrhoea

For better understanding of the pathophysiology of diarrhoea, it is classified as secretory or toxin induced, osmotic or malabsorption induced, inflammatory, iatrogenic/drug-induced, and functional diarrhoea. Most etiologies will have a complex pathophysiology involving one or more of these mentioned mechanisms.

2.8.5 MANAGEMENT OF DIARRHOEA

The mainstay of managing diarrhoeal diseases is determination and correction of fluid depletion (water and electrolyte depletion), shock and acidosis, maintenance of nutrition, and drug therapy (e.g., anti-diarrhoeal agents and antimicrobial therapy). The relative importance of each is governed by the severity and nature of diarrhoea (Singh and Verma, 2012).

2.8.5.1 Non-Pharmacological

Most diarrhoeal illnesses are self-limited and require no specific intervention other than hydration and dietary modification (Lawler and Wallace, 2003).

2.8.5.1.1 Oral Rehydration Therapy (ORT)

Oral rehydration therapy (ORT) is the administration of fluid by mouth to prevent or correct dehydration that is a consequence of diarrhoea. According to Langsten and Hill (1995), it is the cornerstone of treatment to prevent dehydration especially for acute watery diarrhoea. Oral rehydration salt (ORS) solution is the fluid specifically developed for ORT. It simply consists of electrolytes (sodium and potassium chloride) and glucose, which promotes water absorption (De Hostos *et al.*, 2011). A more effective, lower-osmolarity ORS (with reduced concentrations of

sodium and glucose, associated with less vomiting, less stool output, and a reduced need for intravenous infusions in comparison with standard ORS) has been developed for global use (Farthing *et al*., 2008).

2.8.5.1.2 Dietary Modification

Dietary modifications can also significantly alter the course of certain gastrointestinal conditions. For instance, it is evident that avoidance of lactose or gluten-containing foods can greatly benefit patients with lactose intolerance or celiac disease, respectively (Guandalini and Vaziri, 2011). For patients with prominent dumping, dietary modification comprising frequent small, dry meals that are high in protein and low in carbohydrate and substances that prolong the absorption of carbohydrate, such as pectin, may be useful (Mercadante, 1995). Dietary substances that may aggravate IBS symptoms such as fatty foods (which delay stomach emptying but also stimulate the lower bowels leading to bloating and discomfort and diarrhoea), beans and gas producing foods (which can produce bloating and diarrhoea), as well as alcohol and caffeine should be avoided (Drossman *et al.*, 2002).

2.8.5.2 Pharmacological Method

2.8.5.2.1 Antimicrobials

Routine empirical use of antimicrobials in the treatment of acute watery diarrhoea is neither necessary nor appropriate. Antimicrobials should be used only for specific enteric pathogens and a given clinical severity; this approach involves a thoughtful clinical evaluation of each case. *Cholera* and *shigellosis* are among the few gastrointestinal infections in which a specific antimicrobial could meaningfully shorten the disease severity, decrease the risk of complications and reduce its transmission (Salazar-Lindo, 2011).

Antiprotozoal, e.g. nitazoxanide, drugs can be very effective for diarrhoea in children, especially for *Giardia*, *Entamoeba histolytica*, and *Cryptosporidium* (Halsey, 2009). Antimicrobials such as ciprofloxacin and norfloxacin are to be considered the drugs of choice for empirical treatment of traveler's diarrhoea in which ETEC or other bacterial pathogens are likely causes and of community-acquired secretory diarrhoea when the pathogen is known (Akalin, 1993; Guerrant *et al*., 2001). Azithromycin is widely available and has the convenience of single dosing. Treatment for *amoebiasis* should, ideally, include diloxanide furoate following metronidazole, to get rid of cysts that may remain after metronidazole treatment. Trimethoprim-sulfamethoxazole (TMPSMX) is a reasonable alternative to quinolones for empiric treatment of children and patients with sensitivity to quinolones or in areas where quinolone-resistant organisms are prevalent (Guerrant *et al*., 2001). Vancomycin, metronidazole, bacitracin or fusidic acid can also be indicated in severe cases of AAD particularly those related to *Clostridium difficile* (Bergogne-Berezin, 2000; Hogenauer *et al*., 1998).

However, the clinical benefit of antimicrobial therapy must be carefully weighed against the cost, the risk of adverse reactions, harmful eradication of normal intestinal flora, the induction of Shiga toxin production, and the increase of antimicrobial resistance (Guerrant *et al.*, 2001; Lori, 2008).

2.8.5.2.2 Probiotics or Biotherapeutic Agents

Probiotics are live microbial food supplements which beneficially affect the host by improving the intestinal microbial balance. Potentially, probiotics maintain or restore gut microecology during or after antibiotic treatment through the following mechanisms: receptor competition, competition for nutrients, inhibition of epithelial and mucosal adherence of pathogens and translocation (Scaldaferri *et al*., 2012), lower colonic pH thereby favoring the growth of nonpathogenic species (Hempel *et al*., 2012), stimulation of immunity, or production of antimicrobial substances

(Friedman, 2012). These agents have been used in the treatment and prevention of autoimmune autistic disorder (AAD). Most common probiotics include *Bifidobacterium bifidum, Bifidobacterium longum, Lactobacillus acidophilus, Lactobacillus casei* GG, *Lactobacillus bulgaricus, Saccharomyces boulardii* (Bergogne-Berezin, 2000). As probiotics are living organisms given to ill patients, the potential for adverse reactions exists (McFarland, 2006).

2.8.5.2.3 Antimotility and Antisecretory Drugs

In mild diarrhoea, antimotility agents (e.g., loperamide) will lessen stool frequency and, by increasing the time of contact with the gut epithelium, will also lessen stool volume (Field, 2003). They might be recommended in routine noninvasive and noninflammatory diarrhoea, and should be avoided in bloody febrile patients. Loperamide inhibits intestinal peristalsis and has mild antisecretory properties, and is preferred for symptomatic treatment in diarrhoea (Al-Abri *et al.,* 2005). Other drugs such racecedotril (an enkephalinase inhibitor) (Field, 2003), octreotide (somatostatin analogue) (Jensen, 1999), and Crofelemer (CaCCs, Calcium sensitive Chloride Channels inhibitor) (De Hostos *et al.*, 2011) are antisecretory antidiarrhoeal drugs found to inhibit secretion in different diarrhoeal conditions. Selective 5-HT3 receptor antagonists such as cilansetron and alosetron are also effective drugs in relieving pain and normalizing bowel frequency as well as reducing urgency (Drossman *et al.*, 2002).

2.8.5.2.4 Anti-inflammatory Agents

Most of the preparations of this group have actions that inhibit various steps of the arachidonic acid cascade. Some are to decrease the production of many immune and inflammatory mediators that actively inhibit mucosal absorption (Urayama and Chang, 1997). Example of drugs in this group include sulfasalazin (potently inhibits cyclooxygenase (Sharon *et al.*, 1978) and also affect 5-lipoxygenase activity both in intestinal mucosa and neutrophils (Dreyling *et al*., 1987)), zileuton

(a selective inhibitor of 5-lipoxygenase), and glucocorticoids (inhibition of phospholipase A2 activity) (Laursen *et al*., 1994). These drugs are mainly indicated in diarrhoea due to IBDs.

Other groups of drugs like bile acid resins (e.g. cholestyramine), α2 agonists (e.g. clonidine), and proton pump inhibitors (e.g. omeprazol) can also be indicated for treatment of diarrhoea caused by ileal surgery, diabetes mellitus, and gastrinoma syndrome, respectively (Mercadante, 1995). Food supplements such as zinc (Singh and Verma, 2012), folate, vitamin A, magnesium and copper reduce the duration and severity of diarrhoeal episodes in children in developing countries (Kulkarni *et al*., 2012; Sarin, 2012). Supplements like zinc can also have adverse effects such asepigastric pain, lethargy, and fatigue if given in high doses (Lazzerini and Ronfani, 2008). Several adsorbents like cholestyramine, kaolin, pectin, activated charcoal can also be used (Sarin, 2012).

2.8.5.3 Herbal Treatment

Despite immense technological advancement in modern medicine, many people in the developing countries still rely on traditional healing practices and medicinal plants for their daily health care needs (Singh and Verma, 2012). According to World Health Organization (WHO) more than 80% of the world's population relies on traditional medicine for their primary healthcare needs (Hossain *et al.*, 2012). It is often noted that 25% of all drugs prescribed today come from plants. This estimate suggests that plant-derived drugs make up a significant segment of natural product– based pharmaceuticals (Rout *et al*., 2009). Herbal products from medicinal plants are preferred because of less testing time, higher safety, efficiency, cultural acceptability and lesser side effects (Prasad *et al*., 2012).

There have been numerous reports of the use of traditional plants for the treatment of diarrhoeal diseases. The main chemical constituents in plants found to be responsible for anti-diarrhoeal

activity are tannins and tannic acid, flavonoids, alkaloids, sesquiterpenes, diterpenes, terpenes and terpenoids (Sarin, 2012). The chemical compounds present in herbal products are a part of the physiological functions of living organisms, and hence they are believed to have better compatibility with the human body (Prasad *et al*., 2012). Plant extracts can have antispasmodic effects, delay gastrointestinal transit, suppress gut motility, stimulate water adsorption or reduce electrolyte secretion (Palombo, 2006). These activities may explain the benefits of using particular plants in the treatment of diarrhoeal disease. *Acacia catechu* (Sarin, 2012), *Chiranthodendron pentadactylon Larreat* (Velázquez *et al*., 2012), *Bombax buonopozense* (Singh and Verma, 2012), *Croton blanchetianus* Baill, *Croton rhamnifolius* Willd, and *Croton argyroglossum* Baill (Siqueira *et al*., 2012) are few examples, selected randomly, of herbal medicines proven to have anti-diarrhoeal activity.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 PREPARATION OF SAMPLES

The common red variety of sorghum (*Sorghum bicolor*) grains in Nigeria was purchased from local retail market outlet in Mile 12 market, Kosofe, Lagos state. The seeds were authenticated by University of Lagos Herbarium and ascribed a voucher number: LUH 7417. The seeds were carefully freed from foreign materials by hand picking.

Ogi, a gruel was prepared by soaking sorghum grains in water for 3 days followed by wet milling and sieving to remove bran, hulls and germ (Odunfa, 1985). The pomace which was retained on the sieve was discarded while the filtrate was fermented for 2 days to yield *Ogi-baba*, which is sour sediment (Osuntoki and Korie, 2010).

Pito, traditional beverage drink was prepared by soaking sorghum grains in water for 24 hours, followed by malting (saccharification) for 5 days in baskets lined with moistened banana leaves. The malted grains were kilned at 55 ^{o}C for 24 hours to remove moist and hops. The dry grains were wet milled with water (1:1 v/v) and boiled for 4 hours. The resulting mash was allowed to cool and later filtered through a fine mesh basket. The filtrate thus obtained was allowed to stand at ambient temperature and allowed to ferment overnight (Kolawole *et al.*, 2007). *Pito*, the non-alchololic product thus obtained, was a dark brown liquid with characteristic taste.

3.2 ISOLATION AND IDENTIFICATION OF BACTERIAL ISOLATES FROM FERMENTED SORGHUM PRODUCTS

3.2.1 SERIAL DILUTION OF BACTERIAL ISOLATES FROM FERMENTED SORGHUM PRODUCTS

Stock solutions of fermented sorghum products (*ogi-baba* and pito) were serially diluted by dispensing 1ml of well-mixed fermented products into tubes containing 0.1 % peptone water under aseptic conditions and made up to to 10ml with the peptone water. The diluted sample was then used as the base solution to make an additional dilution (100 X dilution) to obtain concentrations of 10^{-2}. Dilution was done to thin out stock solution in order to have sparsely grown colonies of bacterial isolates.

3.2.2 ISOLATION OF BACTERIAL ISOLATES

MRS agar supplemented with 0.01 % (g/v) sodium azide in order to inhibit the growth of Gram-negative bacteria was used. Aliquot (0.2 ml) of 100 X dilution from each sample was transferred into the MRS agar plates, allowed to set and incubated anaerobically at 32 ^{o}C for 48 hours, the different colonies of the bacteria observed. Distinct colonies were randomly isolated and sub-cultured by spread plate technique to purify the strains which were subsequently kept in MRS broths supplemented by 40 % glycerol for further use (Oguntoyinbo and Oni, 2004).

The bacterial isolates were examined by Gram staining for various sizes, shapes, colours and texture, a series of tests such as catalase ammonia production and gas production test. The MRS agar plates were labelled OB for *Ogi-baba* and PB for *Pito.*

3.2.3 MORPHOLOGICAL CHARACTERIZATION AND MICROSCOPIC EXAMINATIONS OF THE BACTERIAL ISOLATES

The isolated colonies of the putative organisms were microscopically examined.

Classifiction of the cultures was initiated by Gram staining: Samples were grown in MRS broths at 37 °C for 24 hours under anaerobic conditions. After incubation cultures were transferred aseptically into 1.5 ml microfuge tubes and centrifuged for 5 min at 6,000 rpm. Then, supernatant was removed and cells were re-suspended in sterile water. Gram staining was performed. Then, under light microscopy Gram positive and purified isolates were determined (Omemu and Faniran, 2011).

3.2.4 INVESTIGATION OF GAS PRODUCTION FROM GLUCOSE BY THE BACTERIAL ISOLATES

In order to determine the homo-fermentative and hetero-fermentative characterization of isolates, CO_2 production from glucose test was done. MRS broths and inverted Durham tubes were prepared and inoculated with 1 % (v/v) overnight fresh cultures. Then the test tubes were incubated at 37 °C for 48 hours. Gas occurrence in Durham tubes was observed during 48 hours which is the evidence for CO_2 production from glucose (Kandler and Weiss, 1986).

3.2.5 CATALASE TEST OF THE BACTERIAL ISOLATES

Catalase test was performed on isolates to observe catalase production.

For this purpose, overnight cultures of isolates were grown in MRS broth under anaerobic conditions. After 24 hours, fresh liquid cultures were used for catalase test by dropping 3 % hydrogen peroxide solution onto 1 ml of overnight cultures. The reaction was allowed to go on for about 2 minutes and examined. The formation of gas bubbles indicates the presence of catalase enzyme (Kandler and Weiss, 1986).

3.2.6 AMMONIA PRODUCTION BY THE BACTERIAL ISOLATES

MRS broths, containing arginine, were used in order to detect ammonia production from arginine. MRS containing 0.3 % L-arginine hydrochloride was transferred into tubes as 5 ml of glucose

solution (1 % w/v) and inoculated with 1 % overnight cultures. Tubes were incubated at 37 °C for 24 hours. After incubation, 100 µl of cultures pipetted into a new petri dish positioned on a white platform. Equal volume of Nessler's reagent was pipetted onto the cultures. The change in the colour was observed. Bright orange colour indicated a positive reaction while yellow indicated a negative reaction. The negative control does not contain arginine (Kandler and Weiss, 1986).

3.3 BIOCHEMICAL CHARACTERIZATION OF THE BACTERIAL ISOLATES

3.3.1 CARBOHYDRATE FERMENTATION

Isolates were characterized according to their fermentation profiles, by the ability to ferment eight (8) different carbohydrates, namely: glucose, xylose, galactose, maltose, lactose, fructose, sucrose and dextrose.

Active cells and sugar solutions were prepared separately in 10 ml MRS broths and incubated at 37 °C for 24 hours. The active cells of isolates were prepared as follow: overnight cultures were centrifuged for 10 minutes at 10,000 rpm. Pellets were washed twice with distilled water and re-suspended in MRS containing pH indicator bromocresol purple (0.04 g/l).

Sugar solutions (100 µg/ml) were prepared as well. The solutions were filter sterilized with filters (0.22 µm pore diameter). The sugar filtrates (400 µl) were pipetted into new tubes containing 1.6 ml of suspended cells. Thus 2 % (w/v) final sugar concentration was obtained.

Also positive and negative controls were used to indicate growth and contamination. A part of suspended cells (1.6 ml) and 400 µl of glucose solution (2 % w/v) were used as positive control while 2 ml of suspended cells was used as negative one (Kandler and Weiss, 1986). After overnight incubation at 37 °C, the turbidity and the colour change from purple to yellow was recorded as positive fermentation results compared with the positive and negative controls.

3.3.2 TEMPERATURE TOLERANCE OF THE BACTERIAL ISOLATES

MRS broths containing bromecresol purple (0.04 g/l) indicator was prepared and inoculated with 1 % of overnight cultures of isolates. The mixtures were incubated for 5 days at 15 °C (refrigeration) and 48 hours at 45 °C (in water-bath). After incubation period at specified temperatures colour change from purple to yellow was observed indicating acid production.

3.3.3 ACID TOLERANCE OF THE BACTERIAL ISOLATES

Isolates were grown in MRS broths at pH 2.0 ± 0.2, previously adjusted using 1M HCl. The MRS broths were inoculated with 1 % (v/v) of overnight cultures of isolates and incubated at 37 ^{o}C for 48 hours. Aliquots of the cultures were taken after incubation period and growth was determined by measuring absorbance (A) at 540 nm. Respective samples were inoculated in MRS (pH 7.0 ± 0.2) as control.

3.3.4 SODIUM CHLORIDE (NaCl) TOLERANCE OF THE BACTERIAL ISOLATES

Isolates were tested for their tolerance to different sodium chloride (NaCl) concentrations. Five ml of nutrient broths (supplemented with 0.5 % dextrose) containing 2 %, 5 % and 10 % NaCl concentrations (w/v) were prepared and 0.04 g/l of bromecresol purple indicator was added. These tubes were inoculated with 1 % overnight cultures and then incubated at 37 °C for 48 hours. The change of the colour from purple to yellow indicated acid production.

3.3.5 INVESTIGATION OF BILE TOLERANCE OF THE BACTERIAL ISOLATES

Isolates were inoculated into MRS broths containing Oxgall (Sigma) of varied concentrations (0.5, 1.0, 1.5 and 2.0 %) and incubated at 37 °C for 24 hours. After incubation period 100 µl of cultures were transferred to MRS agar by pour plate method and incubated at 37 ^{o}C for 24 hours, anaerobically. The growth of isolates on the agar plate was used to confirm isolates as bile salt tolerant.

3.4 INVESTIGATION OF ANTIMICROBIAL ACTIVITIES OF THE BACTERIAL ISOLATES

Nutrient agar was seeded with the indicator organisms (*Escherichia coli, Klebsiella sp., Helicobacter pylori, Bacillus sp., Staphylococcus sp., Salmonella sp., Pseudomonas sp.* and *Listeria monocytogenes*) by inoculating the entire surface of the culture plates and incubated at 37 ^{o}C for 6 hours. Holes (6 mm in diameter) were aseptically punched out of the agar plates, and then, 100 µl overnight MRS broth cultures of the isolates (from fermented sorghum products) were introduced into the holes and incubated aerobically at ambient temperature for 6 hours and anaerobically at 37 ^{o}C for the next 18 hours. After incubation period, inhibitions observed by clear zones extending laterally from the periphery of the holes, containing the isolates, was noted and recorded in mm diameter (Osuntoki *et al.*, 2008).

3.5. DETERMINATION OF ORGANIC COMPOUNDS PRODUCTION BY THE BACTERIAL ISOLATES

The bacterial isolates were grown of MRS broth for 24 hours and samples were taken for lactic acid, hydrogen peroxide and diacetyl production for antibacterial properties

3.5.1 DETERMINATION OF HYDROGEN PEROXIDE PRODUCTION

Diluted sulphuric acid (25 ml) was added to 25 ml of the broth culture of the isolates. Titration was carried out with 0.1 N potassium permanganate 1 ml of 0.1 N potassium permanganate is equivalent to 1.070 mg of hydrogen peroxide. A decolourization of the sample was regarded as end point (AOAC, 1990).

3.5.2 DETERMINATION OF LACTIC ACID PRODUCTION

NaOH (0.1 N) was titrated against 25 ml broth culture of the isolates using 3 drops of phenolphthalein as indicator. The NaOH was added until the colour changes to pink. Each millilitre of NaOH is equivalent to 90.08 mg of lactic acid (AOAC, 1990).

3.5.3 DETERMINATION OF DIACETYL PRODUCTION

Into conical flasks were dispensed 25 ml broth culture of isolates and 7.5 ml of hydroxylamine solution were used for the residual titration. Titration was done with 0.1 N HCl to a greenish end point using bromophenol blue as indicator. The equivalent factor of HCl to diacetyl is 21.5 mg (AOAC, 1990).

3.6 ANTIBIOTIC SUSCEPTIBILITY PROFILE OF THE BACTERIAL ISOLATES

Antibiotic susceptibility of isolates was determined by the ring overlay technique on solid MRS agar at pH 7 with the use of antibiotic multi-discs (MASTRING-S^{TM} M13): chloramphenicol (25 µg), erythromycin (5 µg), Fusidic acid (10 µg), Oxacillin (5 µg), Novobiocin (5 µg), Penecillin G (1 U), Streptomycin (10 µg) and Tetracycline (25 µg) (Mir-hoseini, 2004).

3.7 DETERMINATION OF PROBIOTIC POTENTIALS OF THE BACTERIAL ISOLATES

Each study of the bacterial isolates was scored. The studies considered were antibacterial activities of the isolates against indicator enteropathogens (*Escherichia coli, Klebsiella sp., Helicobacter pylori, Bacillus sp., Staphylococcus sp., Salmonella sp., Pseudomonas sp.* and *Listeria monocytogenes*), tolerance to bile, antibiotic susceptibility and production of lactic acid, acetyl and hydrogen peroxide.

Percentage probiotic potential was calculated as observed score divided by maximum obtainable score as done by Tambekar and Bhutada, 2010.

$$\textbf{Probiotic Potential} = \frac{\textbf{Observed Score}}{\textbf{Maximum Score}} \textbf{ X 100} \quad \textbf{(1)}$$

3.8 GENETIC CHARACTERIZATION OF THE SELECTED BACTERIAL ISOLATES

3.8.1 GENOMIC DNA ISOLATION AND PCR AMPLIFICATION OF 16S rRNA GENE

DNA was isolated from selected isolates grown in 1.5 ml of (OB6 and PB2) MRS broth at 37 °C for 24 hours. The cells were harvested by centrifugation at 10,000 rpm for 5 minutes. After centrifugation, the pellet was collected and washed twice with 1 ml of 0.5 g/l sodium phosphate buffer. 1 ml double distilled water (ddH_2O) was added to the pellet, vortexed and subjected to heating at temperature of 95 °C for 30 minutes. The suspension was then cooled immediately on ice for 30 min and centrifuged at 14,000 rpm for 1 minute. The supernatant was decontaminated with equal volume of phenol : chloroform : isoamyl-alcohol (25 : 24 : 1). DNA was precipitated with absolute ethanol from aqueous phase and washed with 70 % ethanol. The washed DNA was retained for further analyses (Sambrook and Russell, 2001). , DNA pellets were air dried and re-suspended in TE buffer. An amplicon was amplified from the small subunit of 16S rRNA gene using primer set 16S1 (5'-GAGTTTGATCCTGGCTCA-3') and 16S2 (5'-CGGCTACCTTGTTACGACTT-3').

Amplification of 16S rRNA gene was performed in a 25 µl reaction volume containing approximately 50 µg genomic DNA as the template. 5 µl of 0.2 mM deoxynucleoside triphospates, dNTPs (Promega UI20A - UI23A, Madison, WI, USA), 5 µl of 2.5 mM $MgCl_2$, 5 pmol each (0.1 µl volume) of the DNA primer in PCR buffer (Promega, UK), and 5 µl of 1.25 units Taq DNA polymerase (Promega, UK) and 8 µl distilled water. Amplification conditions were as follows:

Table 4: PCR Running Programme

Step	Amplification Condition
1	Placed the tubes in a thermal cycler and set the volume to 10 µl; 1 min 96 °C
2	Repeated this reaction step for 25 cycles:
	96°C for 10 seconds.
	50°C for 5 seconds.
	60°C for 4 minutes.
3	Amplicons removed and forwarded to the sequencing unit

PCR amplification was performed in a BIORAD thermal cycler (BIORAD iCycler, USA). The quality of the extracted DNA was determined by agarose gel electrophoresis1.2 % (w/v), and visualized on UV-transilluminator (Alam *et al.,* 2006).

3.8.2 SEQUENCING AND PHYLOGENETIC ANALYSIS OF 16S rRNA

The PCR product was sequenced in LARAGEN laboratory, by a sequencing reaction mixture (Table 5) and 16S rRNA was obtained. The 16S rRNA sequence of the isolate was compared by alignment against 16S rRNA sequences of LAB available in the Genbank Database using the BLAST. The sequence data was aligned and analyzed to identify the bacterium and its closest neighbors based on the program at: http://www.ncbi.nlm.nih.gov/BLAST (Sukumar and Ghosh, 2010). Phylogenetic relationship among 48 sequences was determined by tree reconstructed using Neighbor-Joining method, incorporated in MEGA 5.2.2 package using Tamura-Nei model (Tamura and Nei, 1993; Tamura *et al.,* 2011).

Table 5: Sequencing Reaction Mixture

Step	Reagents		Quantity
1	Template	single strand DNA (50 - 100 ng)	2 µl
		double strand DNA (200 - 500 ng)	
		PCR product DNA (30 - 90 ng)	
	Primer (5 pmol)		0.5 µl
	BigDye Mix (ABI sequencing Mix)		4 µl
	Deionized water		3.5 µl
	Total Volume		**10 µl**
2	Mixed well and spun briefly in microcentrifuge (Centurion Scientific Ltd, UK)		

Each reaction mix was completed in one tube before starting another and stored in a refrigerator until all mixtures were made.

3.9. β-GALACTOSIDASE ASSAY OF THE SELECTED ISOLATES

Overnight culture was streaked onto MRS agar plate containing 0.01% X-gal (5 Bromo, 4 Chloro-3- indolyl-D-Galactopyranoside) and 0.1 mM IPTG (Hi Media, India) dissolved in Dimethyl sulphoxide (DMSO) as an inducer. The plate was incubated for 24 hours at 37 °C and colour (blue/white) formation was observed for β-galatosidase activity (Sukumar and Ghosh, 2010).

3.10 INVESTIGATION AND CHARACTERIZATION OF ANTIBACTERIAL PROPERTIES OF THE SELECTED BACTERIAL ISOLATES

3.10.1 SCREENING FOR PRODUCTION OF ANTIBACTERIAL AGENT(S)

Selected samples (OB6 and PB2) were cultured in MRS broths and screened for production of antibacterial substance according to the method described by Van Reenen *et al.* (1998). The samples were grown in MRS broths for 18 hours at 37 ^{o}C. The cells were harvested by centrifugation at 10,000 rpm for 5 minutes and the cell-free supernatants adjusted to pH 7.0 with

1 M NaOH. Antimicrobial activity of the selected samples was observed against indicator organism (*E. coli* ATCC25922) by using the agar-well diffusion test method (Olasupo *et al*., 1994).

3.10.2 EXTRACTION OF ANTIBACTERIAL AGENT(S)

Antibacterial agent producing strain (PB2) was cultured in 10 ml MRS broth at 37 °C until early stationary-phase (18 hours old cultures). Cells were harvested by centrifugation at 14,000 rpm for 5 minutes and ammonium sulphate gradually added to the supernatant to 45 % (g/v) saturation. After 4 hours of slow stirring at room temperature, the antibacterial substance was harvested by centrifugation at 14,000 rpm for 30 minutes. Precipitated substance was collected and solubilized in 25 mM ammonium acetate buffer (pH 6.5) (Verkerk *et al.*, 1991).

3.10.3 PARTIAL PURIFICATION OF ANTIBACTERIAL SUBSTANCE EXTRACTED FROM SELECTED ISOLATE

The isolate culture was incubated overnight at 37 °C, and the supernatant was obtained by centrifugation at 10, 000 rpm for 5 min. The pH was adjusted to pH 7 ± 0.2 with 1 N of NaOH and the supernatant cooled in an ice-water bath. Ammonium sulfate was added to the supernatant to a final saturation of 45 % and stirred overnight on a magnetic stirrer. The ammonium sulphate saturated extract was partially purified by chromatography using CM-ion exchanger. Elution was done by the gradient of 10 mM phosphate buffer and 0.1 to 1 M NaCl buffer (pH, 7.0 ± 0.2). Fractions were collected at a flow rate of 0.4 ml/min at 5 minutes interval and optical density (OD) of the fractions was measured at 280 nm (Mehwish *et al.*, 2012)

3.10.4 PROTEIN ASSAY OF ANTIBACTERIAL AGENT(S)

Peptide determination was done by modified Biuret method described by Kingsley (1942). Biuret reagent was prepared by dissolving 3 g $CuSO_4$. $5H_2O$ and 9 g sodium potassium tartrate in 500 ml of 0.2 mol/l NaOH. Potassium iodide (5 g) was added and the volume made up to 1000 ml with

0.2 mol/l NaOH. Bovine serum albumin (1g) was dissolved in 1ml of distilled water (Andrighetto *et al*., 2001). The protein standard was then diluted by mixing with 4 ml distilled water giving a standard protein concentration of 200 µg/ml. This preparation was diluted to prepare the standard solutions of varied concentrations as follows:

Table 6: Preparation of Standard Solutions for Protein Determination

Tube (final conc in µg/ml)	Protein standard (ml)	Distilled Water (ml)
Blank	0.00	5.00
50	1.25	3.75
100	2.50	2.50
150	3.75	1.25
200	5.00	0.00

Biuret reagent (2 ml) was added to 2 ml of standard protein solutions, crude extract, ammonim sulphate extract, partially purified and blank (distilled water). The reactions were placed in a 37 ^{o}C water bath for 10 minutes, cooled to room temperature. The absorbance reading of the cooled solutions were observed at wavelength 540 nm.

3.10.5 DETERMINATION OF MOLECULAR WEIGHT OF THE ANTIBACTERIAL SUBSTANCE EXTRACTED FROM ISOLATE STRAIN PB2 BY SDS-PAGE

The molecular weight of the partially purified protein was determined by 12 % tricine-sodium dodecyl sulfate polyacrylamide gel electrophoresis (Tricine-SDS-PAGE) according to the method of Laemmli (1970) (Shagger, 2006). Sterile glass plates were assembled, 20 ml of 12 % resolving gel were dispensed, 2 ml of butanol was then overlaid onto the gels, allowed to polymerize, after which the overlay was poured off and then the gel surface rinsed with deionized water. To the gel,

8 ml of 4 % stacking gel was overlaid and fixed in an electrophoresis apparatus. To the electrophoresis wells, equal volumes of 1 % SDS (sample buffer) and test samples (crude and partially purified extracts) preheated at 100 °C for 5 minutes and low range molecular weight marker (Dual Xtra standards, BIORAD, USA) respectively were loaded in the gel. The gel was thereafter submerged in running buffer and was initially electrophoresed at 40 Volts for 30 minutes and thereafter at 100 Volts for 120 minutes as described by Schagger and Jargow, (1987). After electrophoresis, the gel was stained with Coomassie® Brilliant Blue R-250 (Saarchem, Krugersdorp, South Africa) and destained by washing overnight with mixture of acetic acid-methyl alcohol-water (5:5:1 v/v).

The distance migrated by the antibacterial substance was noted and used to extrapolate molecular weight as compared with varied distance of migrated by composite proteins of molecular weigh marker (5 – 250 KDa; BIORAD, USA).

3.10.6 DETERMINATION OF ANTIBACTERIAL ACTIVITY OF THE PEPTIDE

The antibacterial activity of the partially purified peptide was quantified by the preparation of 100 µl of twofold serial dilution (100 X dillution) of the peptide in distilled water. The diluent was spotted onto the inoculated lawn of the indicator strain (*E. coli* ATCC25922) and incubated at 37 °C for 24 hours. The peptide activity was estimated as the reciprocal of the highest twofold dilution exhibiting a zone of inhibition and estimated using the formula:

$$\mathbf{1\ AU/ml\ =\ 2^{n}\ X\left(\frac{1000\ \mu l}{V(\mu l)}\right)} \qquad \mathbf{(2)}$$

AU/ml is Arbitrary Unit per milliliter; *n* = highest two-fold dilution showing activity and V = volume used to test antimicrobial activity (Todorov and Dicks, 2009).

3.11 EFFECT OF SURFACTANTS ON ACTIVITY OF THE ANTIBACTERIAL PEPTIDE

The partially purified peptide was adjusted to pH 5.0 with 1 M NaOH. One percent (w/v) of sodium dodecyl sulphate (SDS), urea, Triton X-100 and Ox-gall were added to the peptide separately. Cell-free supernatant without surfactants was used as control. All reactions were incubated at 37 °C for 6 hours and antimicrobial testing was using the agar-well diffusion test method (Olasupo *et al*., 1994).

3.12 EFFECT OF pH ON THE ACTIVITY OF THE ANTIBACERIAL PEPTIDE

The effect of pH on the activity of the peptide was investigated by adjusting the partially purified peptide solution from pH 2.0 to 9.0 with sterile 1 M NaOH or 1 M HCl. After 2 hours of incubation at room temperature (27 °C), the samples were re-adjusted to pH 7.0 with sterile 1 M NaOH or 1 M HCl and antimicrobial testing was done as previously described.

3.13 EFFECT OF TEMPERATURE ON THE ACTIVITY OF THE ANTIBACTERIAL PEPTIDE

The effect of temperature on the pepetide activity was done by incubating the partially purified peptide, adjusted to pH 5, at 20, 30, 37, 40, 60, 80, and 100°C, respectively, for 2 hours. Antimicrobial testing was done as previously described.

3.14 EFFECT OF PROTEINASE AND RNASE ON THE ACTIVITY OF THE ANTIBACTERIAL PEPTIDE

The partially purified peptide was adjusted to pH 5.0 with 1 M NaOH. One millilitre cell-free supernatant was incubated for 2 h in the presence of 1 mg/ml and 0.1 mg/ml of each of proteinase K and RNase (Sigma). Antimicrobial testing was done as previously described.

3.15 EFFECT OF ORGANIC SOLVENTS ON THE ACTIVITY OF THE ANTIBACTERIAL PEPTIDE

The partially purified peptide was treated with 50 % v/v organic solvents including ethanol, phenol, acetone, chloroform and isoamyl alcohol. The mixture was incubated at 37 ^{o}C for 2 hours. After the incubation, the solvent was evaporated by aeration for 30 minutes and antimicrobial activity was assayed as previously described.

3.16 EFFECT OF ULTRA-VIOLET LIGHT ON THE ACTIVITY OF THE ANTIBACTERIAL PEPTIDE

The effect of UV light was studied according to the method of Ogunbanwo *et al.*, (2003). Sterile Petri dishes containing 10 ml crude peptide preparations were exposed to the UV irradiation (UV-transillumination light), placed on the screen. Times of exposure to UV light ranged from 2 to 12 hours. After each 2 hour time interval, peptide activity was estimated as previously described.

3.17. PLASMID PROFILE OF THE ANTIBACTERIAL PEPTIDE PRODUCING ISOLATE

Plasmid DNA of the selected strains (*P. pentosaceus*) was isolated by the method of Anderson and McKay (1983) with some modifications. The preparation protocol was designed to be performed in a 1.5 ml Eppendorf tube. The cells in volume of 1.5 ml of overnight culture were harvested by centrifugation at 8000 rpm for 5 minutes and washed twice with 200 µl of STE buffer (8 % sucrose, 50 mM Tris HCL, 1 mM EDTA, pH=8.0) followed addition of 200 µl freshly prepared lysis solution (1 % SDS, 50 mM Tris HCl, 5 mM EDTA and pH 8.0 adjusted with 5M NaOH) mixed by slow and gentle inversion and incubated at 65 °C for 1 hour. The solution was immediately transferred onto ice for 2 minutes. Thereafter, 100 µl of 5 M NaCl was added to the solution, mixed gently and centrifuged at 12,000 rpm for 10 minutes. The supernatant was transferred to new

microfuge tubes and 600 µl of ice cold ethanol were added, mixed by inversion and placed on ice for 1 hour. The tubes were then centrifuged at 12,000 rpm for 10 minutes minutes and the pellet was washed with 70 % ethanol, dried and dissolved in 20 µl of TE buffer (50 mM Tris and 10 mM EDTA, pH=7) containing RNase A and incubated at 37 ^{o}C for 30 minutes. The extracted DNA was electrophoresed on agarose gel, 0.8 % (w/v) supplemeted with ethidium bromide 0.5 mg/ml, at 70 Volts for 1 hour in TBE buffer (Tris, boric acid and EDTA, pH 8.0). Lambda HindIII DNA molecular mass marker (23130 – 564 bp) was used as a standard for molecular mass determination. The gel was visualized on UV-transilluminator.

3.18 DETERMINATION OF GENES ENCODING THE ANTIBACTERIAL PEPTIDE

DNA was isolated according to the method previously performed (Sambrook and Russell, 2001). Primers Pedpro (5′-CAA GAT CGT TAA CCA GTT T-3′) and Ped 1041 (5′-CCG TTG TTC CCA TAG TCT AA-3′) were designed from the operon encoding pediocin PA-1 (accession number M83924) and synthesised by Genosys Biotechnologies (Europe) Ltd. (Cambridgeshire, United Kingdom) (Todorov and Dicks, 2009).

PCR reactions were performed using a BioRad® PCR (BioRad Thermal Cycler, USA). The following conditions were used: an initial denaturation step of 94°C for 60 seconds, followed by 35 cycles of 94 °C for 60 seconds, 50 °C for 30 seconds and at 72 °C for 60 seconds, and final extension at 72 °C for 5 minutes. The amplified product was visualized in a 1.2 % (w/v) agarose gel stained with ethidium bromide.

3.19 PLASMID CURING OF THE ANTIBACTERIAL PEPTIDE PRODUCING BACTERIAL ISOLATE

Ethidium bromide at concentration 125 µg/ml was added to antibacterial peptide-producing bacterial cells to exert environmental pressures on the cells to foster plasmid loss. The culture was

incubated at 37 °C for 48 hours. After full incubation time, the bacterial cells were assayed for loss of antibacterial peptide activity.

3.20 STATISTICAL ANALYSES

Statistical analysis was performed using SPSS 20.0 software (International Business Machine 'IBM' Corporation, 2014). Data are expressed as mean ± SD for triplicate results.

Genetic data were analyzed using bioinformatics tools such as Basic Local Alignment Search Tool (BLAST) and Molecular Evolutionary Genetics Analysis (MEGA) Version 5.2.2.

All experiments were carried out in triplicates.

CHAPTER FOUR

4.0 RESULTS

4.1 IDENTIFICATION OF LAB ISOLATES FROM FERMENTED SORGHUM PRODUCTS

A total of 11 bacterial strains were isolated from fermented sorghum products. Seven isolates from gruel are labelled OB1 to OB7 and four isolates from beverage which are labelled PB1 to PB4.

4.2 MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF LAB ISOLATES FROM FERMENTED SORGHUM PRODUCTS

The isolates were tentatively identified as six *Lactobacillus* species; three *Pediococcus* species; one *Streptococcus* species and one *Leuconostoc* species based on morphological, phenotypic physiological and biochemical characterization (Table 7). The isolates were characterized to be Gram-positive, catalase-negative and tolerant to 5% (w/v) NaCl. The isolates showed varied capabilities to ferment different sugars as confirmed by acid production (Table 7).

Table 7: Morphological and Biochemical Analyses of Bacterial Isolates from fermented sorghum products (*Ogi-baba* and *Pito*)

Code / **Test**	OB1	OB2	OB3	OB4	OB5	OB6	OB7	PB1	PB2	PB3	PB4
Colour of colony	White	white	White	Pale yellow	White	White	Pale yellow	white	white	White	white
Gram's reaction	+	+	+	+	+	+	+	+	+	+	+
Cell shape	Rod	Rod	Cocci	Cocci	Rod	Rod	Rod	Cocci	Cocci	Rod	Cocci
Catalase	-	-	-	-	-	-	-	-	-	-	-
Gas from Glucose	-	-	+	-	+	-	-	-	-	+	-
Growth at 15^0C	+	+	+	-	+	+	+	-	+	-	+
Growth at 45^0C	-	-	-	+	+	-	+	+	-	+	-
Growth at pH 2.0±0.2	-	-	+	-	+	+	+	+	+	-	+
Growth in 2% NaCl	+	+	+	+	+	+	+	+	+	+	+
Growth in 5% NaCl	+	+	+	+	+	+	+	+	+	+	+
Growth in 10% NaCl	-	-	-	-	-	-	-	-	+	-	-
Arginine Hydrolysis	-	-	-	+	+	-	+	+	+	-	+
Glycerol	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+	+	+
Xylose	-	-	+	+	-	-	+	+	-	-	+
Maltose	+	+	-	+	+	+	+	-	+	-	-
Fructose	+	+	+	+	+	-	+	+	+	+	+
Galactose	+	+	-	+	+	+	+	+	+	+	+
Glucose	-	-	-	-	+	-	-	-	-	+	-
Sucrose	+	+	+	+	+	+	+	+	+	+	+
Dextrose	+	+	-	-	+	+	+	-	+	+	+
Probabl Identity	Lb	Lb	Le	St	Lb	Lb	Lb	Pc	Pc	Lb	Pc

+: positive; -: negative; Lb: *Lactobacillus sp*; Le: *Leuconostoc Sp;* St: *Streptococcus sp;* Pc: *Pediococcus sp.*

4.3 ANTIBACTERIAL ACTIVITIES OF LAB ISOLATES FROM FERMENTED SORGHUM PRODUCTS

The isolated strains exhibited more pronounced antibacterial activities against indicator Gram-positive bacteria, such as *Listeria sp., Bacillus sp.,* and *Staphylococcus sp*., than the indicator Gram-negative bacteria (*E. coli, H. pyloric, Pseudomonas sp., Klebsiella sp.,* and *Salmonella sp.*) (Figure 2).

4.4 PRODUCTION OF ANTIBATERIAL AGENTS BY LAB ISOLATES FROM FERMENTED SORGHUM PRODUCTS

All isolated strains produced lactic acid. However, over 75% of the isolates (OB1, OB2, OB3, OB6, PB1, PB2, PB3 and PB4) produced diacetyl whereas about 55% of isolated strains (OB1, OB6, OB7, PB1, PB2 and PB4) produced hydrogen peroxide (Figure 3).

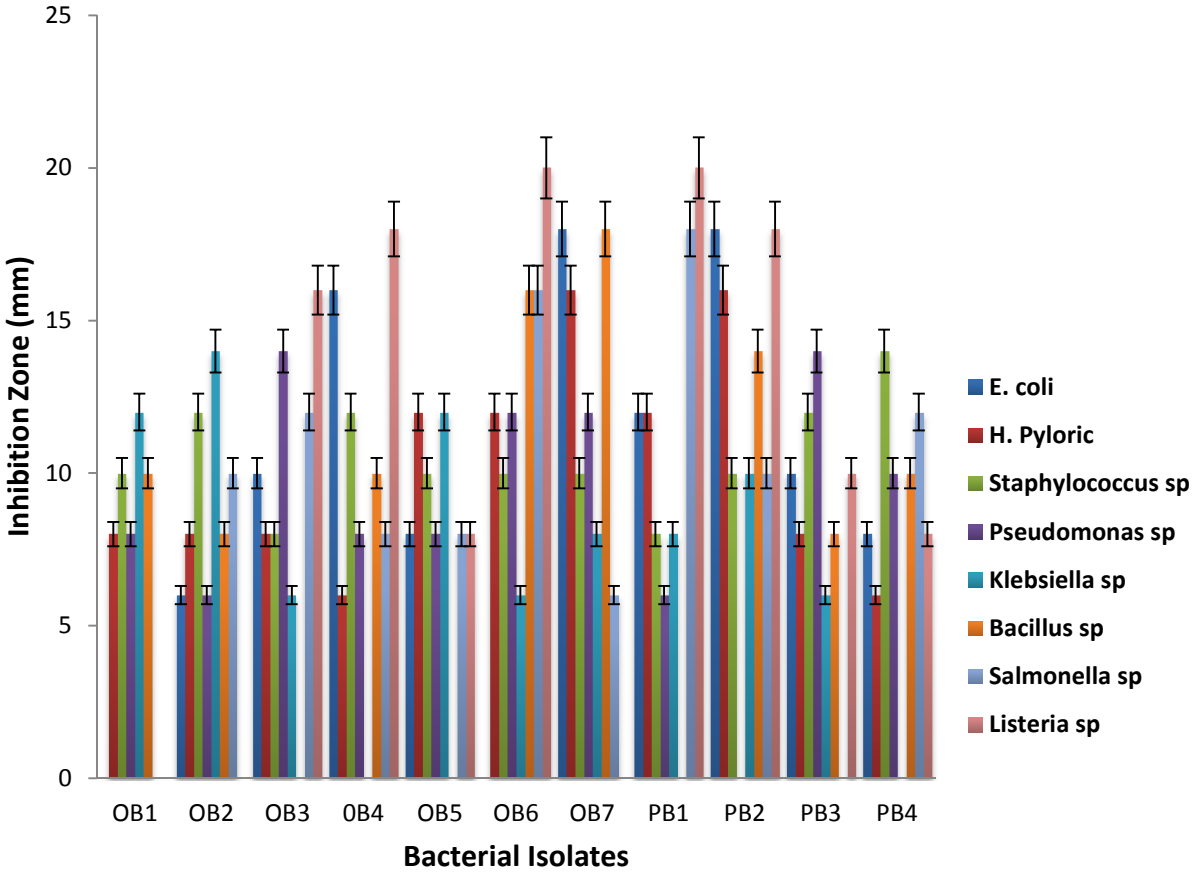

Figure 2: Antibacterial activity of bacterial isolates from fermented sorghum products (*Ogi-baba* and *Pito*) against enteric pathogens

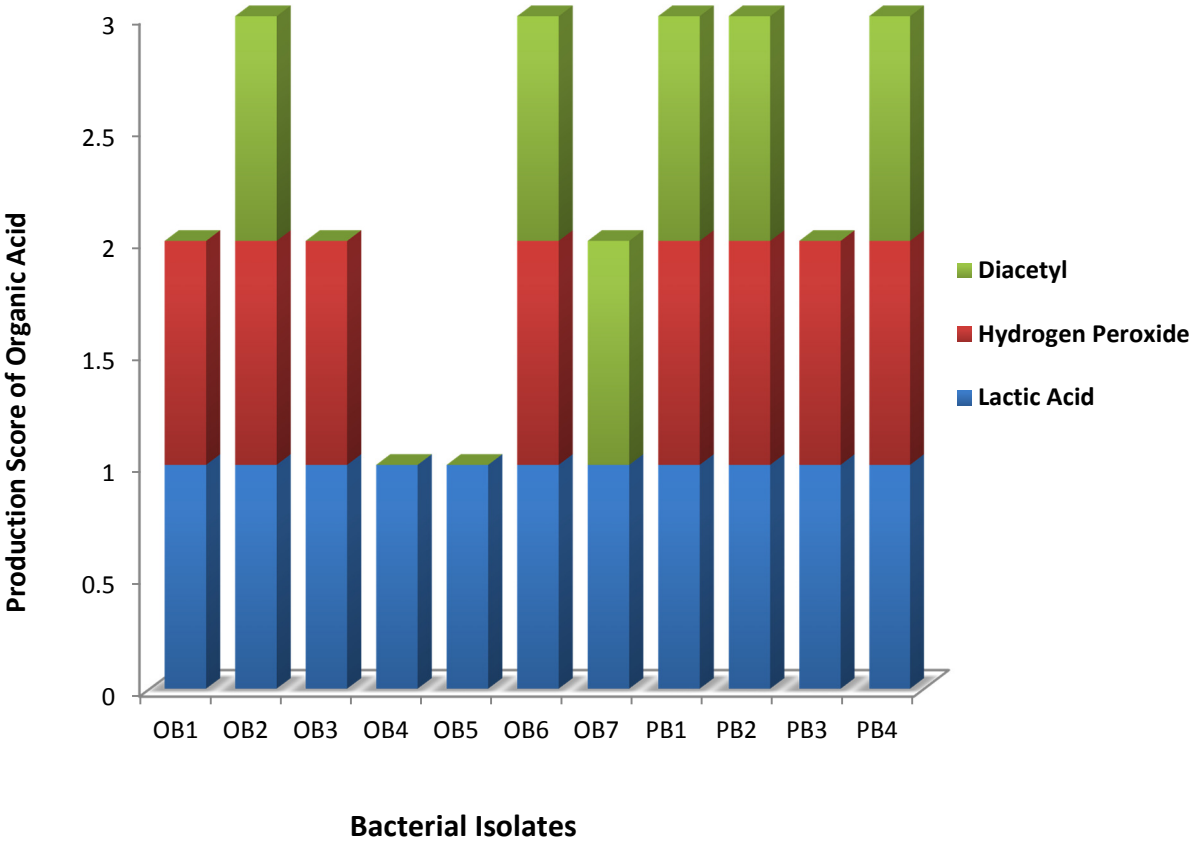

Figure 3: Production of organic compounds by bacterial isolates from fermented sorghum products (*Ogi-baba* and *Pito*)

4.5 ANTIBIOTIC SUSCEPTIBILITY OF LAB ISOLATES FROM FERMENTED SORGHUM PRODUCTS

All isolates demonstrated high susceptibility to Erythromycin 5µg, Fusidic acid 10µg and Penicillin G 1 unit. Isolates OB5 and PB2 exhibited sensitivity to additional antibiotics such as Oxacillin 5µg, Streptomycin 10µg and Tetracycline 25µg. However, all the isolates showed resistance to Chloramphenicol 25µg (Table 8).

4.6 BILE TOLERANCE OF LAB ISOLATES FROM FERMENTED SORGHUM PRODUCTS

Tolerance to bile salts was observed in six isolates (OB5, OB6, OB7, PB1, PB2 and PB4) at 2% (Figure 4).

4.7 PROBIOTIC POTENTIALS OF LAB ISOLATES FROM FERMENTED SORGHUM PRODUCTS

The probiotic potentials of the isolates were calculated and observed to range between 51 and 77.5%; PB2 having highest of 77.42% (Figure 5). The calculations were based on the scoring at different levels of screening for potential probiotic properties, which included bile salt tolerance, organic acid and hydrogen peroxide production, antibiotic susceptibility and antibacterial activities against enteropathogenic indicator organisms.

Table 8: Antibiotic Sensitivity Profiles of Bacterial Isolates from Fermented Sorghum Products (*Ogi-baba* and *Pito*)

Antibiotic	OB1	OB2	OB3	OB4	OB5	OB6	OB7	PB1	PB2	PB3	PB4
Chloramphenicol 25µg	R	R	R	R	R	R	R	R	R	R	R
Erythromycin 5µg	S	S	R	S	S	S	S	S	S	R	S
Fusidic acid 10µg	S	R	S	S	S	S	S	S	S	S	S
Oxacillin 5µg	R	R	R	R	S	R	R	R	R	S	R
Novobiocin 5µg	R	R	R	R	R	R	R	R	S	R	S
Penicillin G 1 unit	S	S	S	R	R	S	S	S	S	S	S
Streptomycin 10µg	R	R	R	S	S	S	S	R	S	R	R
Tetracycline 25µg	R	R	S	S	S	R	R	R	S	R	S
Keys	S: Sensitive; R: Resistant (≥18mm)										

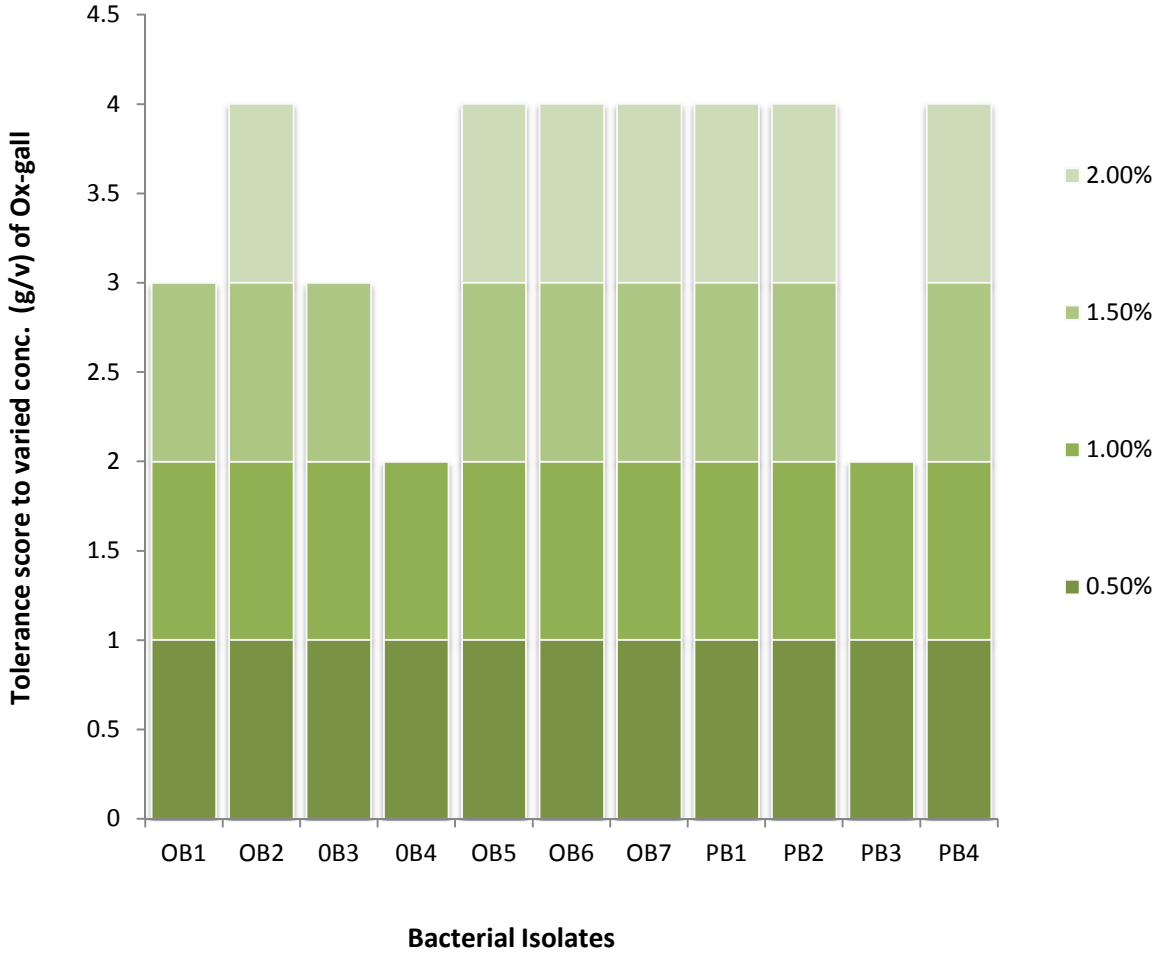

Figure 4: Bile salt tolerance of bacterial isolates from fermented sorghum products (*Ogi-baba* and *Pito*)

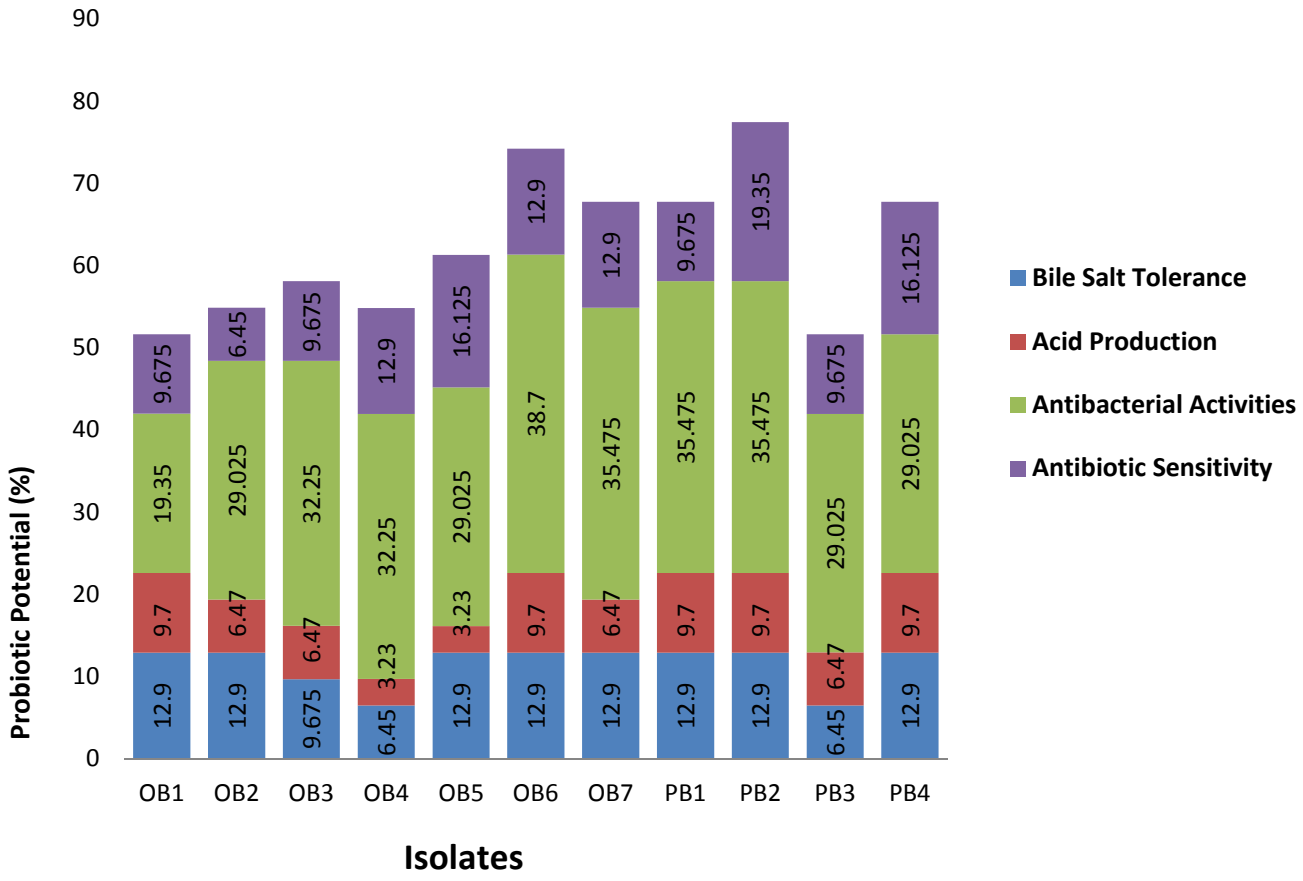

Figure 5: Probiotic potentials of bacterial isolates from fermented sorghum products (*Ogi-baba* and *Pito*)

4.8 16S rRNA SEQUENCE OF OB6 ISOLATE FROM FERMENTED SORGHUM GRUEL 'OGI-BABA'

Figure 6 shows the 16S rRNA gene partial sequence of OB6 after amplification by PCR .The sequence datum of 963 nucleotides was submitted and ascribed ascension number: KP883298.

4.9 MOLECULAR IDENTIFICATION OF OB6 ISOLATE BY BLAST SEARCH

The alignment of 16S rRNA gene partial sequence of OB6 in the Genbank by BLAST run showed 100% identity with *Lactobacillus plantarum* strain 16S ribosomal RNA gene.

4.10 PHYLOGENETIC IDENTIFICATION OF OB6 ISOLATE FROM 'OGI-BABA'

The tree with the highest similar sequences is shown. The tree is drawn to scale, with query sequence (ascension number: KP883298) belonging to OB6 isolate having 100% similarity with three *Lactobacillus plantarum* strains (Figure 7).

>KP883298.1 OB6 16S ribosomal RNA gene, partial sequence

GTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAAC
ATATCTGAGAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCA
GCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTT
TAAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATCGGAAACTGGGAAACTTGAGTGCAGAA
GAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCG
GCTGTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGTATGGGTAGCAAACAGGATTAGATACCCTGGTAG
TCCATGCCGTAAACGATGATTGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTA
AGCATTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGG
TGGAGCATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTGACATACTATGCCAATCTAA
GAGATTAGACGTTCCCTTCGGGGACATGGATACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGA
TGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTATCAGTTGCCAGCATTAAGTTGGGCACTCTGGT
GAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGC
TACACACGTGCTACAATGGATGGTACAACGAGTTGCGAGCTCGCGAGAGTAAGCTAATCTCTTAAAGCCA
TTCTCAGTTCGGACTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTA

Figure 6: 16S rRNA sequence of bacterial isolate OB6 from fermented sorghum product (*Ogi-baba*)

Table 9: BLAST Lineage report of OB6 isolaated from fermented sorghum product (*Ogi-baba*)

Closest Genetic Match	**% of Similarity**
Lactobacillus plantarum	100
Lactobacillus pentosus	99
Lactobacillus vaccinostercus	96
Lactobacillus diolivorans	94
Lactobacillus kisonensis	94

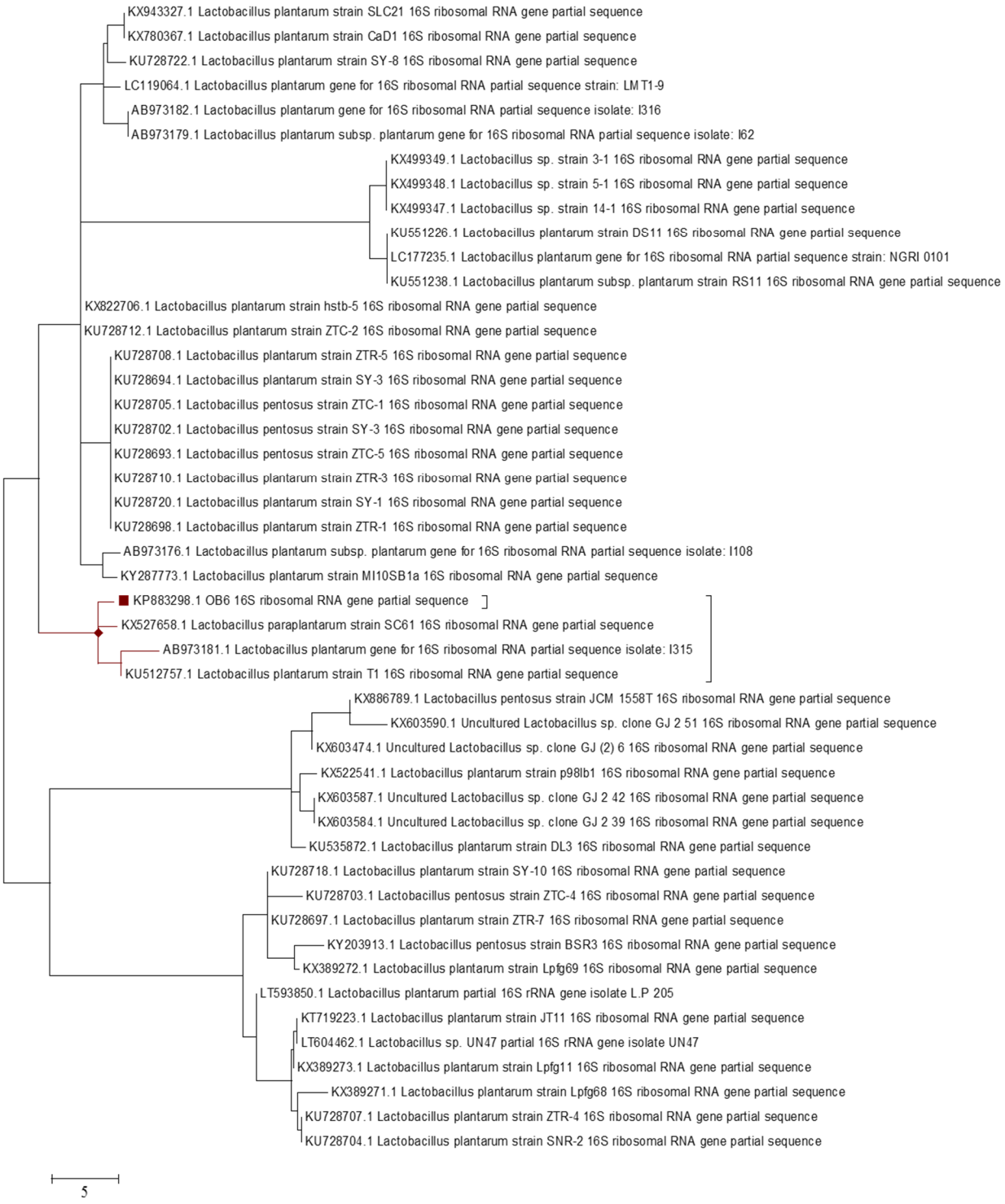

Figure 7: Molecular Phylogenetic analysis of of OB6 isolated from fermented sorghum product (*Ogi-baba*) by Maximum Likelihood method

4.11 16S rRNA SEQUENCE OF PB2 ISOLATE FROM FERMENTED SORGHUM BEVERAGE 'PITO'

Figure 8 shows the 16S rRNA gene partial sequene of PB2 after amplification by PCR .The sequence datum of 966 nucleotides was submitted and ascribed ascension number: KP883297.

4.12 MOLECULAR IDENTIFICATION OF PB2 ISOLATE BY BLAST SEARCH

The alignment of 16S rRNA gene partial sequence of PB2 in the Genbank by BLAST run showed 100% identity with *Pediococcus pentosaceus* strain 16S ribosomal RNA gene.

4.13 PHYLOGENETIC IDENTIFICATION OF PB2 ISOLATE FROM 'PITO'

The tree with the highest similar sequences is shown. The tree is drawn to scale, with query sequence (ascension number: KP883297) belonging to PB2 isolate having 100% similarity with four *Pediocccus pentosaceus* strains (Figure 9).

>KP883297.1 PB2 16S ribosomal RNA gene, partial sequence

CTGATGGAGCACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAGCTCTGTTGTTAAAGAAGAACGTG
GGTAAGAGTAACTGTTTACCCAGTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCC
GCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTCTTTTAA
GTCTAATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATTGGAAACTGGGAGACTTGAGTGCAGAAGAG
GACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCT
GTCTGGTCTGCAACTGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCC
ATGCCGTAAACGATGATTACTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGT
AATCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAAGAATTGACGGGGGCCCGCACAAGCGGTGG
AGCATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTGACATCTTCTGACAGTCTAAGAG
ATTAGAGGTTCCCTTCGGGGACAGAATGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGT
TGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTACTAGTTGCCAGCATTAAGTTGGGCACTCTAGTGAG
ACTGCCGGTGACAAACCGGAGGAAGGTGGGGACGACGTCAAATCATCATGCCCCTTATGACCTGGGCTAC
ACACGTGCTACAATGGATGGTACAACGAGTCGCGAGACCGCGAGGTTAAGCTAATCTCTTAAAACCATTC
TCAGTTCGGACTGTAGGCTGCAACTCGCCTACACGAAGTCGGAATCGCTAGTAATC

Figure 8: 16S rRNA sequence of bacterial isolate PB2 from fermented sorghum product (*Pito*)

Table 10: BLAST Lineage report of PB2 isolated from fermented sorghum product (*Pito*)

Closest Genetic Match	**% of Similarity**
Pedicoccus pentosaceus	100
Pedicoccus acidililactici	99
Pedicoccus damnosus	96
Pedicoccus ethanolidurans	97
Pedicoccus pavulus	95
Lactobacillus kisonensis	95

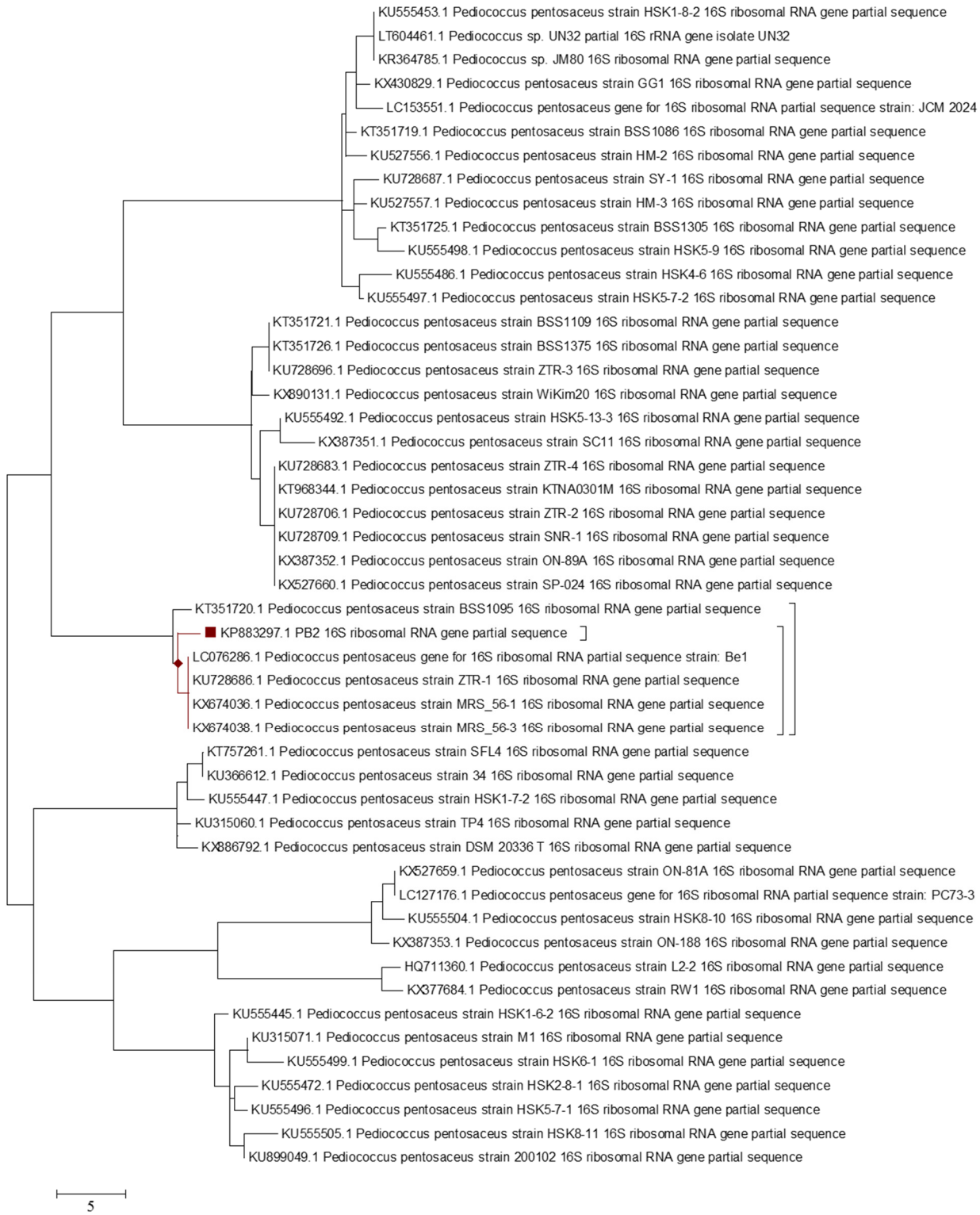

Figure 9: Molecular Phylogenetic analysis of PB2 isolate from fermented sorghum products (*Pito*) by Maximum Likelihood method

4.14 β-GALACTOSIDASE ASSAY OF P. PENTOSACEUS PB2 FROM PITO

Both strains (*L. plantarum* OB6 and *P. pentosaceus* PB2) showed blue/green colonies on MRS agar supplemented with X-GAL (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG (isopropyl β-D-1-thiogalactopyranoside) and thus β-galactosidase activity (Plate 1).

The two strains (OB6 and PB2) produced β-galactosidase which hydrolyzed chromogenic X-gal in the presence and inducer, IPTG, to form 5-bromo-4-chloro-indoxyl, which spontaneously dimerized to produce an insoluble blue/green pigment called 5,5'-dibromo-4,4'-dichloro-indigo (Plate 1).

4.15 ANTIBACTERIAL PROTEINACEOUS SUBSTANCE PRODUCTION BY PB2 ISOLATE FROM PITO`

Pediococcus pentosaceus PB2 was observed to inhibit of growth of standard indicator *Escherichia coli* ATCC25922 whereas *Lactobacillus plantarum* OB6 showed no activity. The PB2 strain exhibited secretion of antibacterial proteinaceous substance (APS) (Plate 2).

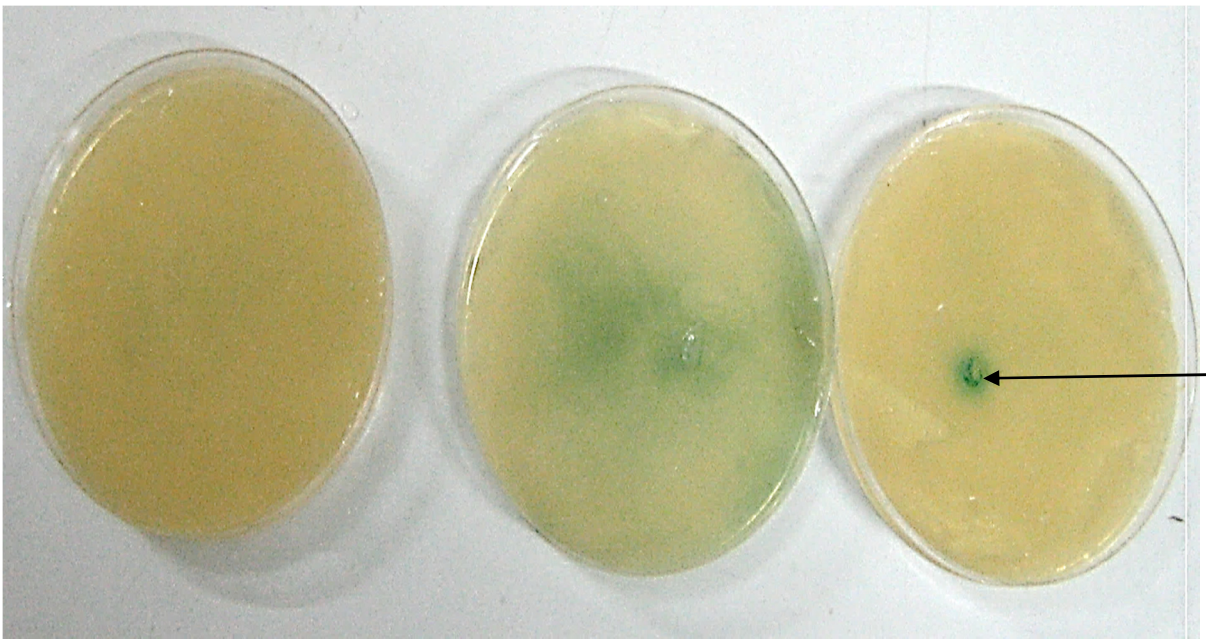

Plate 1: β-Galactosidase activity of selected strains OB6 and PB2 isolated from fermented sorghum products (*Ogi-baba* and *Pito*)

OB6: *L. plantarum* OB6 grown on nutrient agar supplemented with X-gal;

PB2: *P. pentosaceus* PB2 grown on nutrient agar supplemented with X-gal;

Ctrl: nutrient agar supplemented with X-gal without bacterial isolate

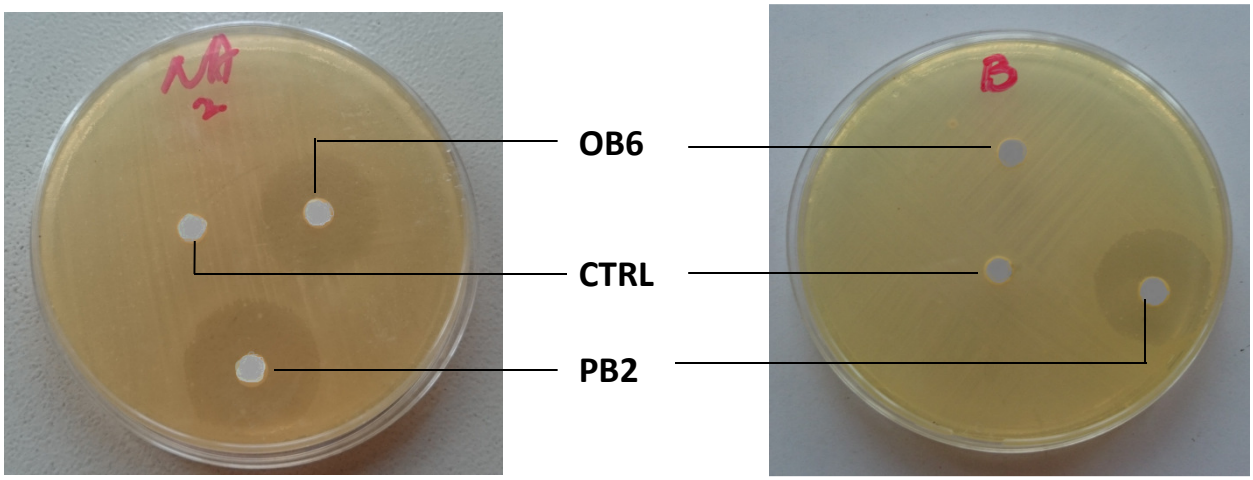

Plate 2: Antibacterial activity of selected isolates from fermented sorghum products (*Ogi-baba* and *Pito*): Plate NA = Nutrient Agar inoculated with test isolates; Plate B = Bacteriocin screening agar inoculated with test isolates

OB6 = *L. plantarum;* PB2 = *P. pentosaceus* and CTRL = MRS broth without bacterial cells.

4.16 PURIFICATION TABLE OF ANTIBACTERIAL PROTEINACEOUS EXTRACT OF PB2 FROM PITO

The results of the purification procedures are summarized in table 11. After final purification step, the proteinaceous substance from *P. pentosaceus* PB2 was purified 12.62-fold with a recovery of 13.3%. The partially purified substance has molecular weight 4.87 kDa (Plate 3).

4.17 EXTRACTION AND PARTIAL PURIFICATION OF ANTIBACTERIAL PROTEINACEOUS SUBSTANCE FROM PB2 ISOLATE FROM PITO

The elution profile of the extracted proteinaceous substance from *P. pentosaceus* PB2 following partial purification by cation exchange (Carboxymethyl Sepharose CM-50 column) showed antibacterial activity in fractions from 31 to 41 (2ml each) (Figure 10).

The partially purified active fractions (31 – 41) were pooled together and subjected to SDS-PAGE. The result indicated that the proteinaceous substance was approximately 4.87KDa (Plate 3) indicating that the antibacterial substance is a peptide.

Table 11: Purification profile of the antibacterial proteinaceous substance

PURIFICATION STEPS	VOLUME (ml)	MIC (mg/ml)	TOTAL PROTEIN (mg)	SPECIFIC ACTIVITY (UA/mg)	TOTAL ACTIVITY (UA)	RECOVERY YEILD (%)	PURIFICATION FOLD
Crude Extract	*100*	*10*	*48.3*	*0.21*	*1,000.00*	*100*	*1.00*
Ammonium Sulphate(45) precitation	*18*	*22.22*	*17.35*	*1.28*	*399.96*	*40.0*	*6.10*
Carboxymethyl-Sephadex C-50 chromatography	*24*	*5.56*	*2.10*	*2.65*	*133.44*	*13.3*	*12.62*

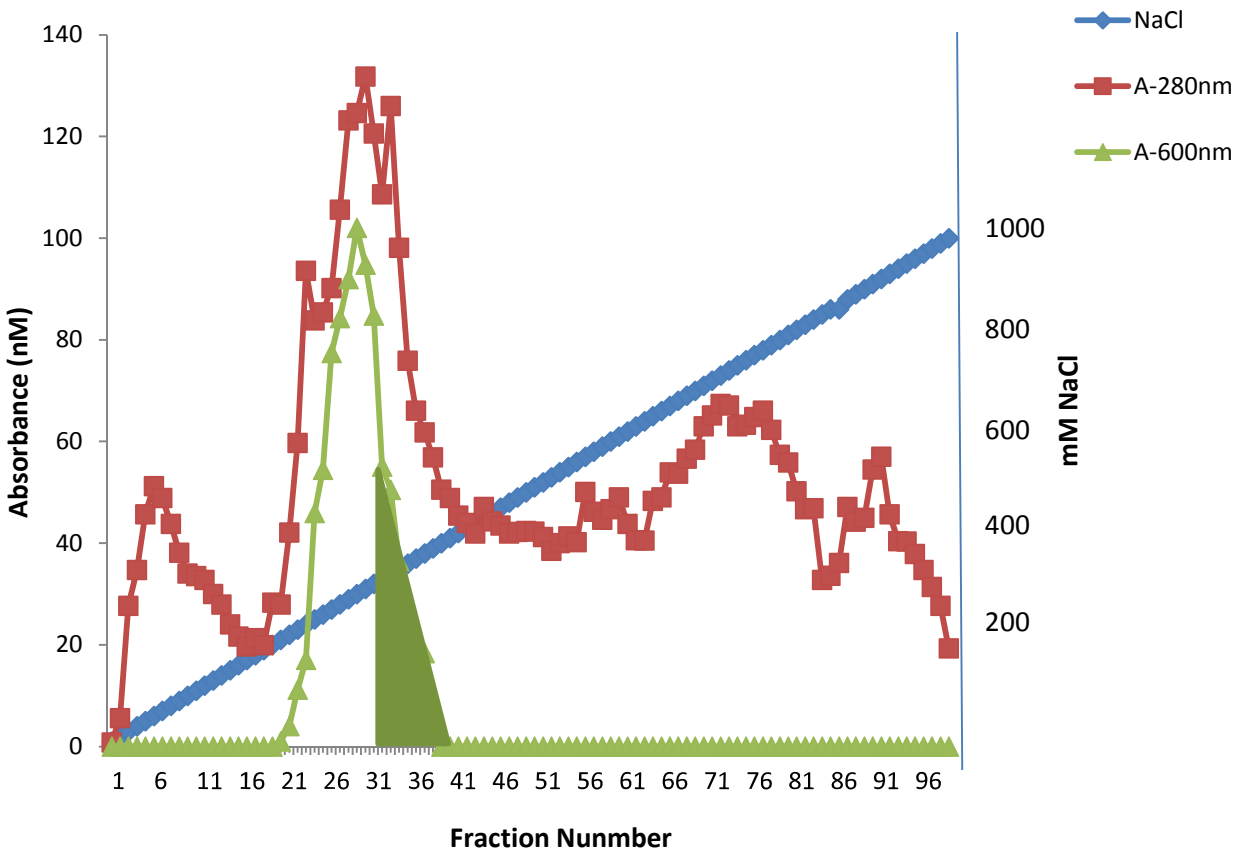

Figure 10: Elution Profile on CM-Sephadex C-50 of the antibacterial proteinaceous substance from *P. pentosaceus* PB2

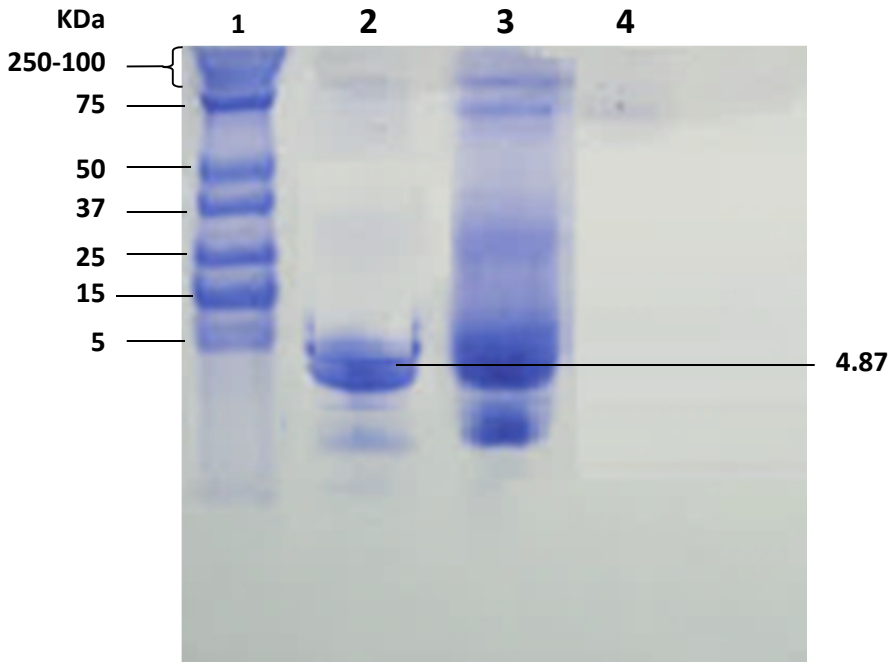

Plate 3: SDS-Polyacrylamide gel electrophoresis of the antibacterial proteinaceous substance from *P. pentosaceus* PB2, stained with Coomassie brilliant Blue.

Lane 1: molecular weight marker (5 – 250 KDa; BIORAD, USA);

Lane 2: active eluent of cation exchange chromatograph (1);

Lane 3: peptide bands of crude extract (2);

Lane 4: Phospate buffer without peptide (Control).

4.18 OPTIMAL CONDITIONS FOR THE ACTIVITY OF THE ANTIBACTERIAL PEPTIDE OF PB2 ISOLATE FROM PITO

The partially purified proteinaceous substance showed optimal activity against both *E. coli* ATCC25922 and *L. monocytogenes* at pH values of 5.0 (Figure 11) and temperature of 40^{o}C (Figure 12).

4.19 EFFECTS OF ORGANIC SOLVENTS ON THE ACTIVITY OF THE ANTIBACTERIAL PEPTIDE OF PB2 ISOLATE FROM PITO

Analyses of the effects of organic solvents on the antibacterial activity of the peptide showed reduction of activity leaving over 50% activity against both indicator organisms (Figure 13).

4.20 EFFECTS OF SURFACTANTS AND ENZYMES ON THE ACTIVITY OF THE ANTIBACTERIAL PEPTIDE OF PB2 ISOLATE FROM PITO

The antibacterial activity of the peptide was lost when treated with proteinase K while some activity was observed when treated with RNase and surfactants (Figure 14).

4.21 EFFECTS OF UV ILLUMINATION ON THE ACTIVITY OF THE ANTIBACTERIAL PEPTIDE OF PB2 ISOLATE FROM PITO

The study of influence of UV light on the substance activity showed loss of activity after 12 hr exposure to irradiation (Figure 15).

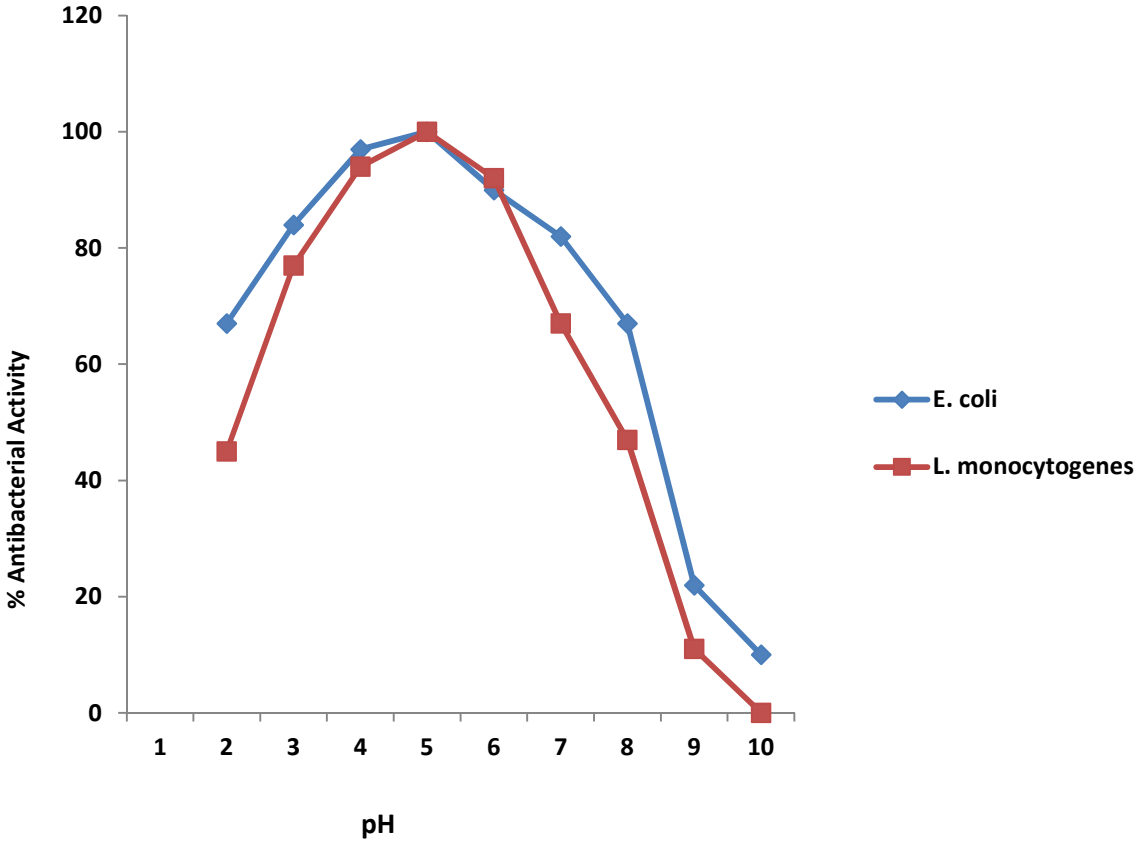

Figure 11: Effect of pH on inhibitory activity of the the antibacterial peptide against *E. ccoli* and *L. monocytogenes*

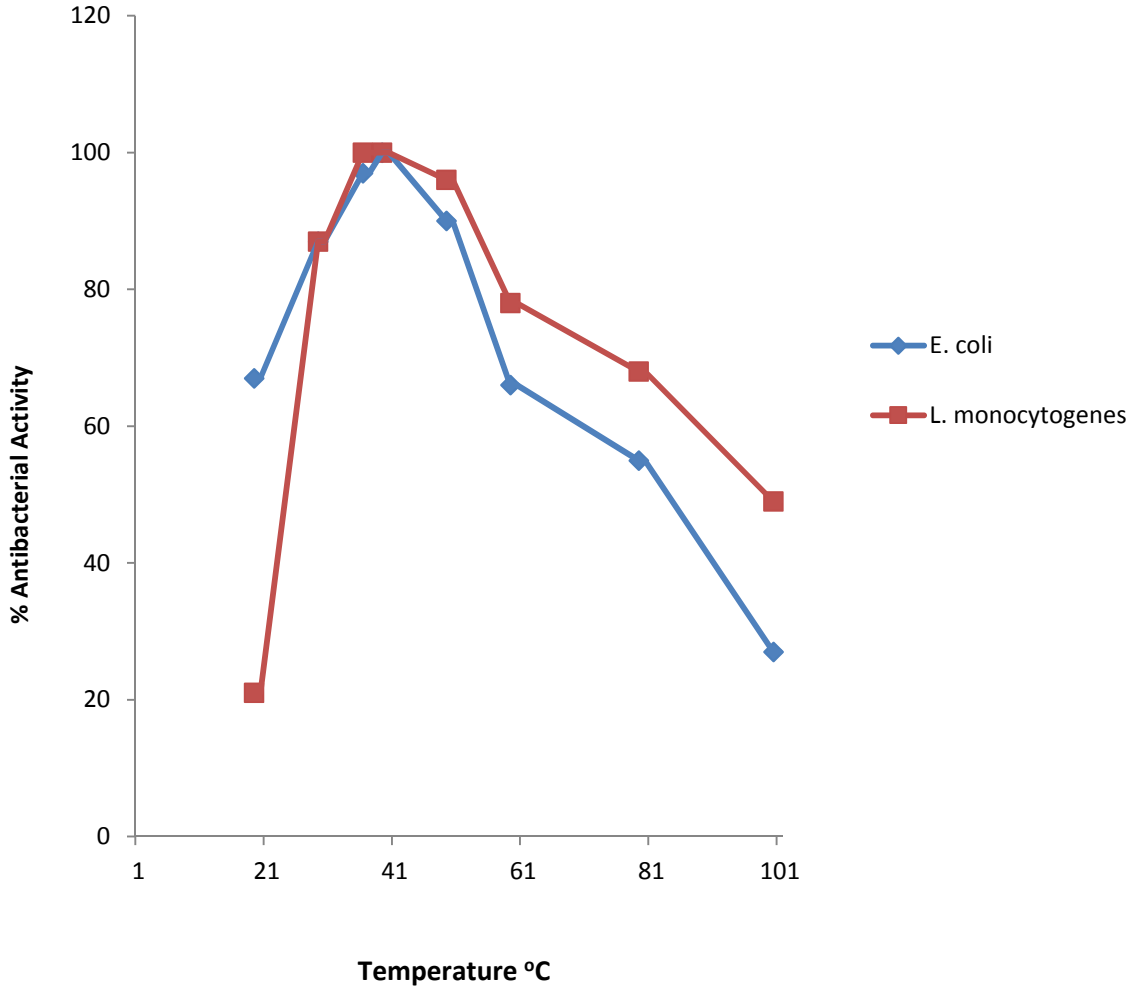

Figure 12: Effect of temperature on activity of the antibacterial peptide against *E. coli* and *L. monocytogenes*

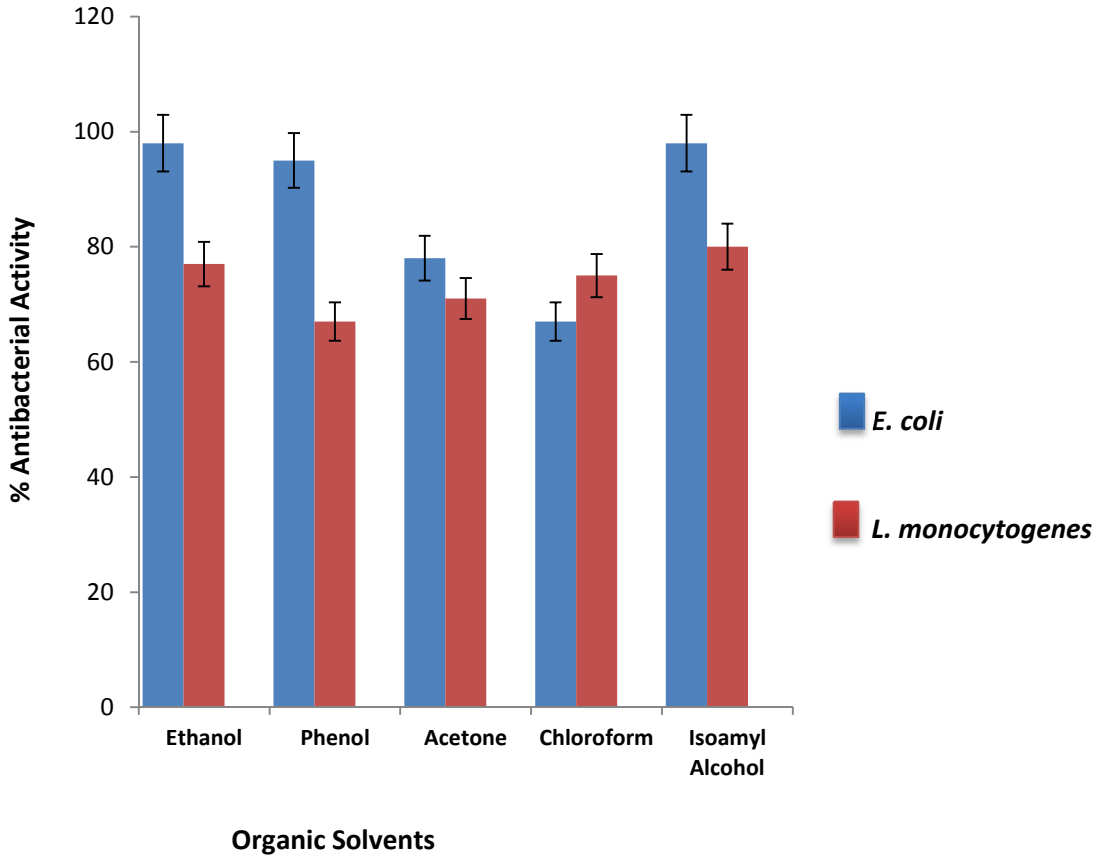

Figure. 13: Effect of organic solvents on activity of the antibacterial peptide against *E. coli* and *L. monocytogenes*

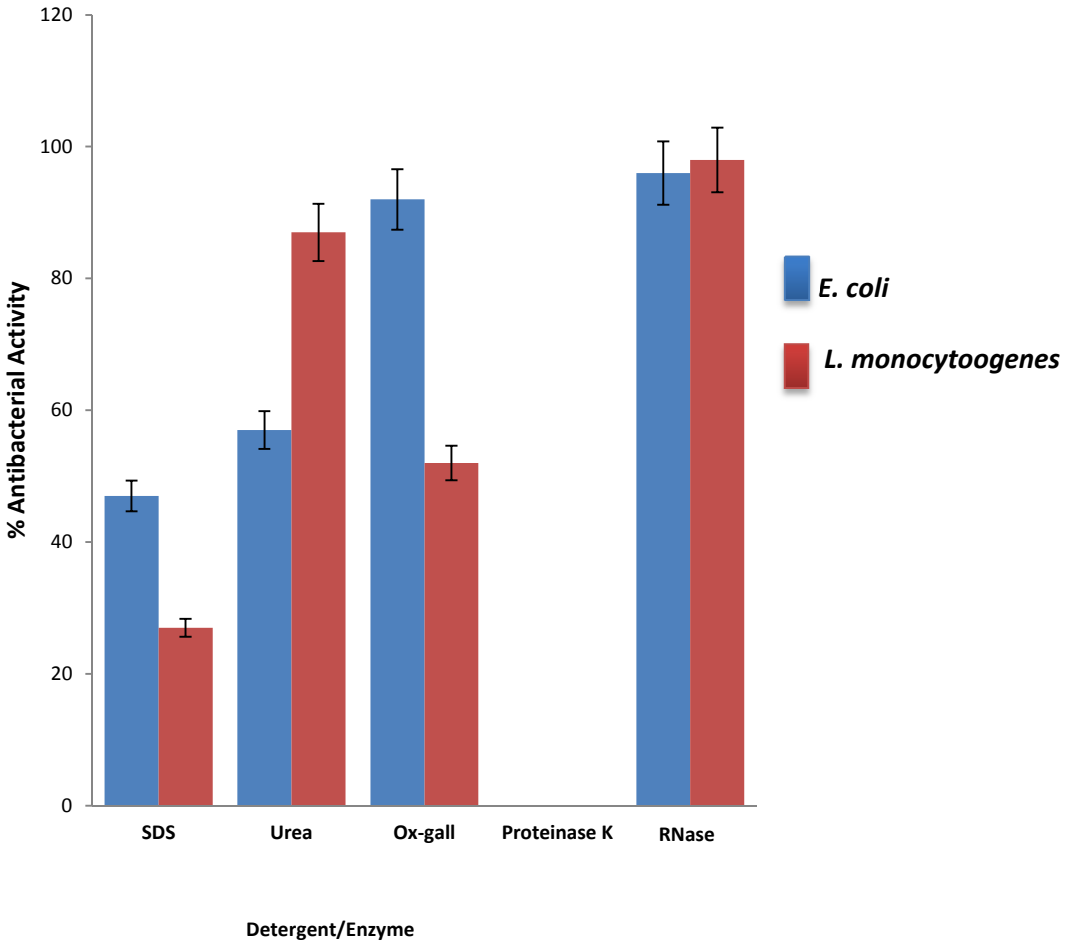

Figure 14: Effects of surfacants and enzymes on activity of the antibacterial peptide against *E. coli* and *L. monocytogenes*

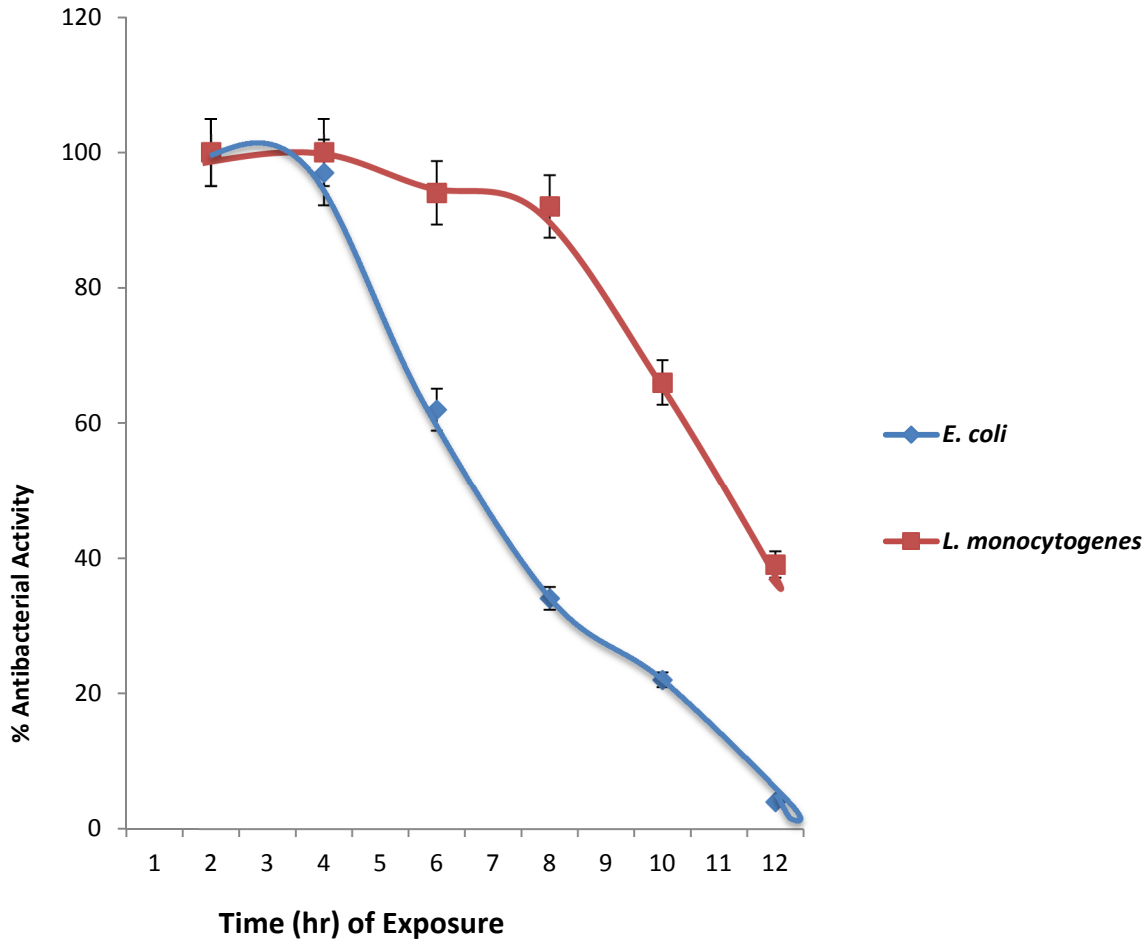

Figure 15: Effect of UV illumination on activity of the antibacterial peptide against *E. coli* and *L. monocytogenes*

4.22 MOLECULAL DETERMINATION OF THE ANTIBACTERIAL PEPTIDE GENE OF PB2 FROM PITO

Molecular analysis showed that the gene encoding the protenaceous substance is 727 basepairs in size (Plate 4).

4.23 GENE EXPRESSION OF THE ANTIBACTERIAL PEPTIDE OF PB2 FROM PITO

The result obtained with the curing experiment (Plate 5) showed that expression of gene for the antibacterial protenaceuos substance produced by *P. pentosaceus* PB2 was chromosomal as antibacterial activity was obseved against *E.coli* and *L. monocytogenes* after plasmid curing of PB2 (Plate 6).

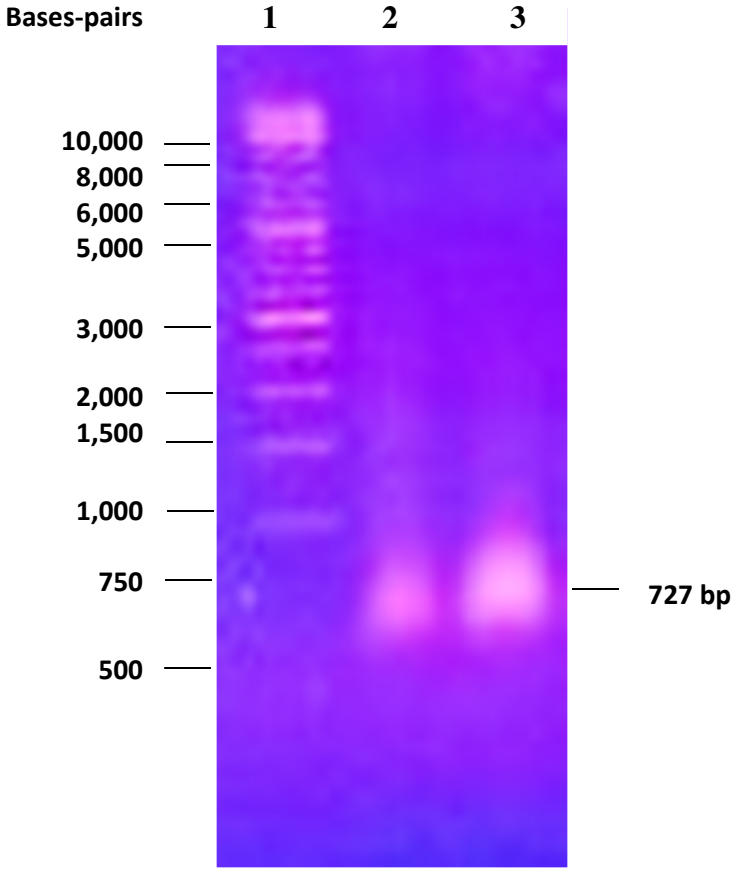

Plate 4: Amplification of gene encoding antibacterial pepetide gene of *P. pentosaceus* PB2 with specie-specific primers Pedpro and Ped 1041.

Lane 1: molecular weight marker;

Lanes 2 and 3: *P. pentosaceus* PB2

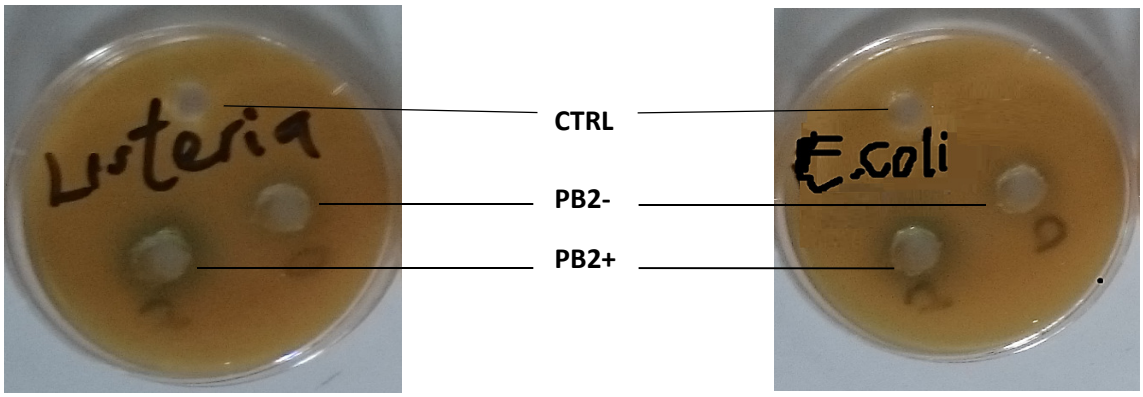

Plate 5: Curing *P. pentosaceus* PB2 of bacteriocin activity

PB2+ = Wild; PB2- = Cured; CTRL = Control (double distilled water)

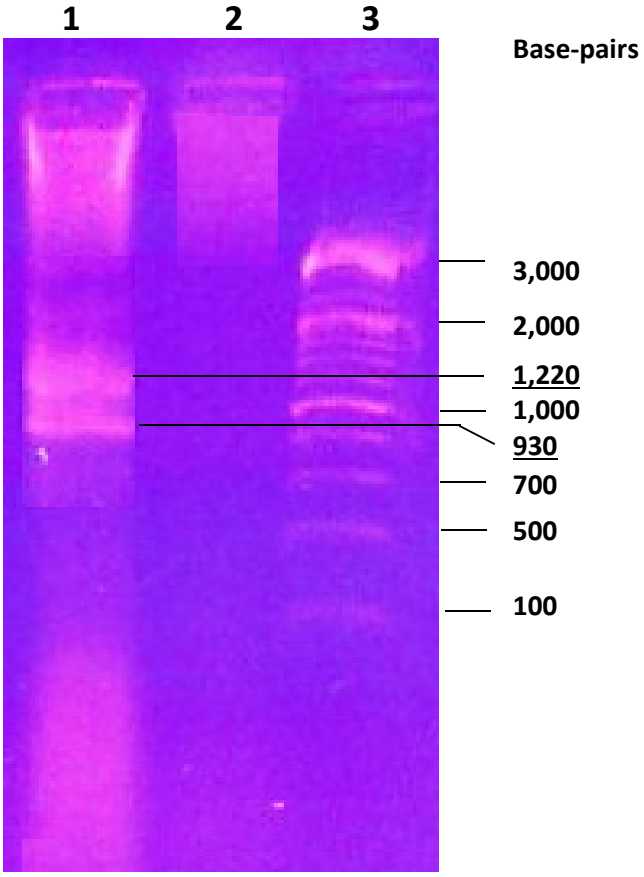

Plate 6: Plamid profile and curing *P. pentosaceus* PB2 of antibacteria activity.

Lane 1: PB2+ = PB2

Lane 2: PB2- = Cured PB2

Lane 3: MWt. = λ HindIII molecular mass marrker

CHAPTER FIVE

5.1 DISCUSSION

Enteropathogenic bacteria are of global public health concern because of the associated significant morbidity and mortality especially in low and middle income countries where they place tremendous burdens on fragile health care systems (Denno and Paul, 2017).

Though strains of *Lactobacillus* species which are most employed as probiotics (Ruiz *et al.*, 2013) were found to predominate amongst the population of bacteria isolated from fermented sorghum the homo-fermentative nature of *Pediococcus pentosaceus* strain (PB2) indicates that it will grow substantially faster than other bacteria present in the same ecological niche. This may enhance very rapid domination and establishment in a wide range of environments and will be advantageous in the development of a probiotic (Hugenholtz, 2008).

The observation that PB2 tolerated temperature as high as 45^{o}C showed the ability to grow at high temperature which is a desirable trait because a high fermentation temperature could reduce contamination by other microorganisms less tolerant to heat (Mohd Adnan and Tan, 2007).

Additionally, strain PB2 was tolerant to high NaCl (10% w/v) concentration. Bacteria adapt to hyper-osmolarity by accumulation, synthesis and transport of compatible solutes to restore turgor. It has also been well documented that osmo-protectants could play additional positive roles and have beneficial effects on membrane integrity, thermo-protection, protein folding and stability (Baliarda *et al.*, 2003) this could also be a contributory factor to the thermo-tolerant ability of PB2.

The tolerance of some of the isolated strains (including PB2) to acidity and bile salt is vital for bacterial survival and growth in the gastro-intestinal tract. These attributes are the main

requirements for bacteria to be considered probiotics (Dunne *et al.,* 2001). Tolerance to bile salts is a prerequisite for colonization and metabolic activity of bacteria in the small intestine of the host (Collins *et al.,* 1998). This will help probiotics to reach the small intestine and colon and contribute in balancing the intestinal micro-flora (Tambekar and Bhutada, 2010).

This study also found the antibacterial properties of the isolated strains against enteropathogenic bacteria that are of clinical importance. The antimicrobial properties of the isolated LAB were detected to have resulted from production of lactic acid, diacetyl and hydrogen peroxide. In addition to production of organic acids and hydrogen peroxide PB2 produced other anti-enterogenic agent(s). The preservative effect exerted by LAB is mainly due to the production of organic acids (such as lactic, acetic, propionic acids) which result in lowered pH (Daeschel, 1989) and production of other compounds including carbon dioxide, hydrogen peroxide, diacetyl and bacteriocins among others. The production of acids lowers the pH of the digestive tract and inhibits the growth of the pathogenic microorganisms (Sukumar and Ghosh, 2010).

Antibiotic resistance of enteric LAB is of major concern because they are highly amenable to conjugation (Clewell and Weaver, 1989) and also successful donor organisms for the transfer of antibiotic resistance genes to unrelated enterococci (Rice *et al.*, 1998), lactobacilli (Shrago and Dobrogosz, 1988), other Gram-positives including *Bacillus subtilis* (Christie *et al.*, 1987), *Staphylococcus* (Young *et al.*, 1987) and *Listeria* spp. (Charpentier and Courvalin, 1997); and even Gram negative bacteria (Courvalin, 1994; Vally *et al.,* 2012). Hence, sensitivity of PB2 to majority of tested antibiotics makes it potentially useful as a probiotic and an alternative to antibiotic therapy and this reduces the potential transfer of genetic materials between it and other GIT microbes. The PB2 that is able to produce antibacterial peptide may also be useful as a probiotic

against identified resistant strains of enteric pathogens that are resistant to selected antibiotics to which PB2 is resistant.

β- Galactosidase activity reduces lactose intolerance and is an important probiotic property (Sukumar and Ghosh, 2010). Therefore, the expression β- Galactosidase activity by PB2 and a strain of *Lactobacillus plantarum* (OB6) indicates potential use in the management of lactose intolerance.

The production of antimicrobial compound other than organic acids by *Pediococcus pentosaceus* was confirmed by a bacteriocin screening medium. The rate of recovery and purification fold obtained for the antibacterial substance were similr to those described for some bacteriocins (Papagianni and Papamichael, 2011). The purification of the peptide demonstrated a successful step in of chromatography with the antibacterial activity of the eluent. The use of low saturation of ammonium sulphate to precipitate the peptide combined with filtration chromatography and electrophoresis suggested aggregate of low molecular weight as observed for other bacteriocins (De Vuyst and Leroy, 2007). The presenc of amino acids with a polar side might be associated with the formation of the aggregates (Teixeira *et al.,* 2013).

The elution profile from the cation exchange chromatography indicated that the pepetide presents a weak residual negative charge because it was eluted with low ionic strength (≤ 0.5mM NaCl). This fact also suggests the presence of a polar amino acid side chain(s) containing a carboxyl group (Teixeira *et al.,* 2013)

PB2 exhibited growth inhibitory activity against *Escherichia coli* ATCC25922. Activity against Gram-negative bacteria is an unusual phenomenon in *Pediococcus* species (De Kwaadsteniet *et*

al., 2005; Todorov and Dicks, 2009; Gong *et al.*, 2010). This has been attributed to the lipopolysaccharide layer of the cell wall protecting the cell membrane in Gram negative, the site of action of bacteriocins (Stevens *et al.*, 1991). The inhibition of *E. coli* is very relevant since the organism is one of the major pathogens responsible for the increasing mortality in children lower than five years old (Ballal and Shivananda, 2002).

The purified antibacterial substance which was proteinaceous in nature was found to be active against *E. coli* and *L. monocytogenes* which are considered to be important food borne pathogens.

The antibacterial peptide produced by PB2 was completely inactivated by proteinase K which is in contrast to a report of Todorov and Dicks (2009) which reports slightly resistance to proteolytic enzymes. Proteinaceous nature of the antibacterial substance was also confirmed by loss of activity on to UV irradiation (Ogunbanwo *et al*., 2003). The sensitivity of peptide to UV irradiation indicated the presence aromatic amino acids were revealed by UV irradiation and the antimicrobial activity was sensitive to the treatment with proteinase K (Teixeira *et al.,* 2013).

The antibacterial peptide was sensitive to chloroform and some other organic solvents. This observation is highly suggestive of the presence of a lipid moiety in the antibacterial peptides according to Wilson and Goulding (1986). Reduction of activity when exposed to chloroform for a long duration indicates the presence of lipid moiety in a protein.

Based on the result obtained with the curing analysis, the production of antibacterial peptide by the *P. pentosaceus* strain PB2 is chromosomally mediated. Bacteriocin production may be either plasmid encoded (Gonzalez and Kunka, 1987) or chromosomally encoded (Holck *et al.*, 1994).

The findings of this study indicate that the antibacterial peptide produced by *Pediococcus pentosaceus* PB2 is a lipopeptide as confirmed by reduction in activity in the presence of organic solvents, like chroloform, with broad spectrum of activity against Gram-positive bacteria and Gram-negative bacterial species. Thus this study indicates that the antibacterial peptide may represent a potential alternative to combat enteric pathogens. These results also reinforce the importance of *Pediococcus pentosaceus* PB6 as a source of a variety of antimicrobial substance(s). The identification and characterization of novel stain PB2 and its potential use in the control of microbial infections are topics of greatest relevance.

5.2 SUMMARY OF FINDINGS

S/N	Objective	Finding(s)
1	To isolate and identify putative beneficial bacteria from fermented sorghum products (*Ogi-baba* and *Pito*)	11 isolates with evidence of lactic acid production were obtained from the two fermented sorghum products (gruel and beverage) with evidence of lactic acid production. The isolates were identified to belong to the genera of Lactobacillus, Pediococcus, Streptococcus and Leuconostoc.
2	To examine isolates for probiotic properties.	*Lactobacillus* sp (OB6) and *Pediococcus* sp (PB2) with cumulative probiotic scores ≥ 70%, were selected based on acidity tolerance, bile salt tolerance, antibiotic susceptibility, organic acid and hydrogen peroxide production and antibacterial activities.
3	To characterize and genetically identify selected isolate(s).	The two selected strains were identified by 16S rRNA sequencing and homology alignment as *Lactobacillus plantarum* OB6 and *Pediococcus pentosaceus* PB2. The sequences for the two isolates were submitted to GenBank and were ascribed ascension numbers: KP883298 and KP883297, respectively. The two isolates demonstrated the ability to hydrolyze lactose.

4	To investigate and characterize the basis of antagonistic activity of identified isolate(s)	The antibacterial nature of *L. plantarum* was found to be as a result of secretion of organic acids and hydrogen peroxide whereas *P. pentosaceus* produced an antibacterial peptide which had inhibitory effects on both Gram-positive and Gram-negative enteropathogens. The peptide was active optimally at 40^{o}C and pH 5. Its activity was negatively affected by organic solvents and surfactants. The gene encoding the antibacterial peptide, produced by *P. pentosaceus* was found to be chromosomal.

5.3 CONTRIBUTIONS TO KNOWLEDGE

1. This study isolated two strains of lactic acid bacteria; *Pediococcus pentosaceus* PB2 and *Lactobacillus plantarum* OB6, with probiotic properties from fermented sorghum products.
2. The research established the sequence data of the 16S rRNA of the two probiotic organisms, *Pediococcus pentosaceus* PB2 and *Lactobacillus plantarum* OB6 that had been deposited in the GenBank and ascribed ascension numbers: KP883297 and KP883298 respectively.
3. The work revealed the strain of *Pediococcus pentosaceus* PB2 had a broad spectrum antibacterial property associated with a 4.87KDa peptide which is chromosomal borne.

5.4 CONCLUSION AND RECOMMENDATIONS

Fermentation is a major food processing technique that enhances the nutritive value of the products and at the same time preserving the fermented foods by microbial activities. Fermentation as practiced in the developing countries is a low-cost and appropriate technique for producing staple diets. The beneficial attributes of microbes involved in local fermentation offer exploitable means for the improvement of local fermented foods.

This study has generated useful information on the antibacterial activity of a strain of *Pediococcus pentosaceus* PB2 isolated from fermented sorghum and has opened up a vista of possible approaches to solving the problems of bacterial contamination of fermented foods.

Antibacterial producing *Pediococcus* species are required to enhance the shelf life of fermented foods predisposed to bacterial contamination by retarding and subsequently preventing the growth of the contaminants. This will impede spoilage and reduce or eliminate the risk of GIT disorders.

The overall effect will be an enhanced shelf life and improve food safety which will increase the ambition towards food security.

Biochemical and genetic studies discovered production of proteinaceous extract with antibacterial activity. The proteinaceous extract also known as bacteriocin may find use as a preservative agent or confer probiotic potential(s) on microbes. It may also be helpful in the development and design of new antibacterial agent.

The findings of this research are expected to significantly contribute to the improvement of the quality of local foods. This will increase the acceptability and increase the derivable income from these foods.

Consequently, due to consumers demand for the locally fermented foods like *ogi* and *pito*, the bacteriocin producing bacterial strains are considered a potential source of biological preservatives for such locally fermented foods.

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