RAFFINOSE METABOLISM AND UTILISATION BY *L. PLANTARUM* ISOLATED FROM INDIGENOUSLY FERMENTED CEREAL GRUELS FOR NUTRITIONAL IMPROVEMENT

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ABSTRACT

Most African foods used in weaning are usually cereal-based gruels fermented by Lactic Acid Bacteria (LAB). The food mainly supplies carbohydrate; excessive intake of which might cause malnutrition in growing children. Minimum dietary requirements of a child could be met through fortification with protein-rich supplements. Soybean is rich in dietary protein but contains some antinutritional factors and raffinose, an oligosaccharide responsible for gas formation, bloating and flatulence in weaning children. The use of microorganisms for hydrolysing raffinose has not been fully exploited in Nigeria. In this study, the use of LAB to hydrolyse raffinose, reduce antinutritional factors and improve nutritional composition of such food blends were investigated.

Commercially- hawked "Ogi" (CO) samples, Local Varieties (LV) of sorghum and maize were obtained from Bodija market, Ibadan and Typed Varieties (TV)-Samsorg 40, Samsorg 41 and Ex-Kano from the Institute of Agricultural Research and Training, Ibadan. The LAB were isolated from spontaneously-fermenting cereal gruels and identified using standard methods. Nine strains of *Lactobacillus plantarum* were selected based on the abundant production of α-galactosidase, and characterized by PCR amplification of 16SrDNA genes. Plasmid presence was determined using agarose gel electrophoresis and the effect of plasmid curing was monitored. The growth of the organisms and metabolites production in different carbon sources were monitored at 20^0C to 80^0C and pH of 3.0 to 9.6. Soyabean was pre-treated by milling, cooking and roasting while the relationship of the isolates to raffinose metabolism during fermentation was monitored daily for 5 days. Reducing sugar, residual oligosaccharides, nutritional, antinutritional factors and alpha-galactosidase were determined using UV-spectrophotometer following Association of Official Analytical Chemist procedures**.** Data were analysed using ANOVA at p=0.05.

One hundred and twenty LAB isolates were obtained and identified as *L. plantarum (35.8 %), L. fermentum (12.5 %), L. pentosus (7.5 %), L. acidophilus (15.8 %), L. casei* (5.8 %), *L. brevis (6.7 %), L. cellobiosus (6.7 %), L. jensenii (5.0 %) and L. reuterii (4.2 %).* Analyses of the nine *L. plantarum* isolates revealed high sequence identities (97.0 %). These isolates exhibited significant differences in utilization of

raffinose at varying concentrations of 0.2 -1.0 mg/mL, while isolates obtained from LV performed better than those from TV and CO. Fermentation reduced the oligosaccharide content of the soyabean by 74.6 % while the reducing sugars increased by 65.0 %. Fortification of the gruel with soyabeans using uncured *L. plantarum* strains improved the nutritional quality (protein: 8.4 to 17.8 %, fat: 3.6 to 12.9 %, ash: 2.0 to 3.8 %, Fe: 6.4 to 10.7 mg/100g and Ca: 156.7 to 211.0 mg/100g), and a significant reduction in antinutritional factors (Tannin: 1.9 to 0.1 mg/g, Phytate: 1.2 to 0.1 mg/g and Trypsin Inhibitor : 1.2 to 0.0 mg/g) was observed after fermentation . Oligosaccharide content, reducing sugar, nutritional and antinutritional composition and organoleptic attributes of the end product were significantly affected by plasmid curing.

Utilisation of raffinose by *Lactobacillus plantarum* from local food sources reduced antinutritional factors and oligosaccharides in soybeans. Nutritional quality of cereal gruels were improved by inclusion of *Lactobacillus plantarum.*

Keywords: Lactic acid bacteria, Raffinose, Plasmid curing, Soybeans and weaning foods.

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CERTIFICATION

I certify that this research study was carried out by STELLA MOJISOLA ADEYEMO in the Department of Microbiology, Faculty of Science, and University of Ibadan, Nigeria under my supervision.

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DEDICATION

This work is dedicated first, to the Glory of The Almighty God who has been my source of inspiration and help throughout my academic career. Also, to my family that has always been there for me at all times and to all nursing mothers along the West African Coast.

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CHAPTER ONE

1.0 INTRODUCTION

Fermentation has been a major way of preserving food in the last several thousands of years. Microbial growth either of natural or inoculated population cause chemical, biological or textural changes in foods. They also form products that can be stored for extended periods; the fermentation process also is to create food with pleasant flavours, aroma, taste, texture, odours and other desirable characteristics (Wakil and Onilude, 2009). The major fermentation type used in the food industry is the lactic fermentation by Lactic Acid Bacteria (LAB). These LAB are usually isolated from fermented foods and beverages while also being found in decomposing plants and lactic products. They produce lactic acid as the major metabolic end-product of carbohydrate fermentation. This trait has, throughout history, linked LAB with food fermentations, as acidification inhibits the growth of spoilage agents (Holzapfel and Wood, 1998; Amankwah *et al.*, 2012).

LAB are a group of bacteria characterized by their ability to synthesize lactic acid. Typical LAB are gram-positive, non-sporing, catalase-negative, lacking cytochromes. They are anaerobic but tolerate little amount of oxygen. They can be rod or cocci and they produce lactic acid as the major end product of fermentative metabolism (Axelssson, 2004). Proteinaceous bacteriocins are also produced by several LAB strains and provide an additional hurdle for spoilage and pathogenic microorganisms. Furthermore, lactic acid and other metabolic products contribute to organoleptic and textural profile of the food item (Holzapfel and Wood, 1998). The industrial importance of the LAB is further evidenced by their reputation as generally regarded to as safe (GRAS) status, due to their ubiquitous appearance in food and their contribution to human health (Nes *et al*., 1996). LAB are characterized by an increased tolerance to a lower pH range which partially enables LAB to outcompete other bacteria in a natural fermentation, as they can withstand the increased acidity from organic acid production, for example, lactic acid and acetic acid (Wakil and Onilude, 2009).

As a result of their use as probiotics in food, LAB have been implicated in several fermented foods used as weaning foods (Nagai *et al.*, 2012). Spontaneous lactic acid fermentation of cereal products is the cheapest method of safely preparing and preserving weaning foods, the contamination of which is a major cause of disease in young children and associated nutrient deficiency and malnutrition (FAO/WHO 1995, 1997). Also, the

metabolism and physiology of LAB is used in different biotechnological processes in industries to formulate LAB starters with useful metabolic activities and capabilities so as to ensure a wide range of quality fermented products with consistent characteristics (Ammor *et al.*, 2006).

In many West African countries, exclusive breastfeeding is usually adequate up to three to four months of age, but after this period, it may become increasingly inadequate to support the nutritional demand of the growing infant. Thus, in a systematic weaning process, there is always the need to introduce soft, easily swallowed foods to supplement the infant's feeding early in life (Onofiok and Nnanyelugo, 1998; Mariam, 2002).

In Nigeria, the usual first weaning food is called pap, *akamu*, *ogi* or *koko*, made from fermented cereal such as maize (*Zea mays*), millet (*Pennisetum americanum*), or guinea corn (*Sorghum spp*.)(Onofiok and Nnanyelugo, 1998). Such weaning foods are prepared traditionally by first hand-picking the dirt or stones in the cereal and washing with water. This is then followed by soaking in water, wet milling and fermentation (Rambouts and Nout, 1995). In Nigerian *'Ogi'*, maize, millet or sorghum grains are washed and steeped for 24 to 72 hours during which they undergo lactic acid fermentation (Banigo and Muller, 1972). They are then drained, wet-milled and finally sieved to yield fine smooth slurry with about 8% solid content and high water content (Banigo and Muller, 1972).

Such weaning foods are usually high in carbohydrate content and are of low nutritive value. They are characterized by low protein, low energy density and high bulk (Onofiok and Nnanyelugo, 1998). Cereal-based diets have been found to be of lower nutritional value than animal-based ones and this forms the primary basis for most of the traditional weaning foods in West Africa. The protein content of maize and guinea-corn is of poor quality, low in lysine and tryptophan. These two amino acids are indispensable to the growth of the young child (Fashakin and Ogunsola, 1982). The problems inherent in the traditional weaning foods and feeding practices in West Africa predispose the infant to malnutrition, growth retardation, infection and high mortality (Guiro *et al*., 1987).

Legumes are rarely used for weaning because of their associated problems of indigestibility, flatulence, diarrhoea and discomfort in children (Townsend and Pitchford, 2012). They may, however, be introduced later in life (after 6months) or be used as food blend to supplement or fortify the fermented cereal nutritionally (Badamosi *et al.*, 1995).

Food fortification or supplementation is the public health policy of adding micronutrients (essential trace elements and vitamins) to foodstuff to ensure that minimum dietary requirements are met (Mariam, 2002; Nagai *et al.*, 2012). Addition of micronutrients to staples and condiments can prevent large-scale deficiency disease and prevent the attended cases of malnutrition that is associated with using cereal based diet as weaning food (Malleshi *et al.*, 1989). Several types of food supplements have been recognized including additives which repair a deficit to "normal" levels or additives which appear to enhance a food and supplements taken in addition to the normal diet.

A dietary supplement, also known as food supplement or nutritional supplement, is a preparation intended to provide nutrients, such as vitamins, minerals, fiber, fatty acids, proteins or amino acids that are missing or are not consumed in sufficient quantity in a person's diet. Some countries define dietary supplements as foods, while in others they are defined as drugs (http://en.wikipedia.org/wiki/Dietary_supplement).

Supplements containing vitamins or dietary minerals are included as a category of food in the Codex Alimentarius, a collection of internationally recognized standards, codes of practice, guidelines and other recommendations relating to foods, food production and food safety. These texts are drawn up by the Codex Alimentarius Commission, an organization that is sponsored by the Food and Agriculture Organization (FAO) of the United Nations (UN) and the World Health Organization (WHO). The Food Supplements Directive of 2002 requires that supplements be demonstrated to be safe, both in quantity and quality (Nagai *et al.*, 2012; http://en.wikipedia.org/wiki/Dietary_supplement).

Soybean is one of such legumes that are used to supply additional nutrients and vitamins to cereal-based weaning food. It is rich in high level of dietary protein and it is easily accessible to the local populace (Castillo *et al.*, 1996). It has been used by several authors to formulate food blend that are rich in protein and used as weaning food in different part of Nigeria. It is thus able to supply the other nutrients that are lacking in such food (Fashakin and Ogunsola, 1982; Fashakin and Awayefa, 1986; Badamosi *et al.*, 1995). Also, Onilude *et al.* (1999) formulated infant weaning foods from fermented blends of cereal and soybeans using *Lactobacillus plantarum* and *Saccharomyces cerevisae* as starter organisms.

Soybean utilization is, however, limited because it contains some anti-nutritional factors. There is also the presence of some oligosaccharides such as raffinose, stachyose and verbascose which possess flatulence-inducing properties in children (Castillo *et al.*,

1996; Townsend and Pitchford, 2012). This problem has been solved by various authors in the past by dehulling, cooking, wet or dry milling, addition of sugar and oil, use of chemicals, germination and fermentation (Fashakin and Ogunsola, 1982; Fashakin and Awayefa, 1986; Onofiok and Nnanyelugo, 1998).Some of these processes, however, may alter the composition of the weaning food, some are time and energy consuming, while others may be expensive or difficult to preserve. Thus, there has been agitation over the years to look for simple fermentation technology approach in order to arrive at nutritionally adequate ready-to-feed weaning foods that can be preserved safely without getting contaminated (Malleshi *et al.,* 1989; Nagai *et al.*, 2012).

This work is, based on the use of *Lactobacillus plantarum* to hydrolyze the oligosaccharide raffinose that is present in soybeans by lactic fermentation. *L.plantarum* is able to break down the complex sugar in this food substance into simple sugars and make it readily available to infants in food that it is added as a supplement (Gray *et al.,* 2012). The organism is also able to reduce the anti-nutrients that are present in the soybeans to levels that are safe for consumption by young children and also improve the nutritional composition of cereal-based gruels that are used as weaning food (Gray *et al.,* 2012).

Local raw materials were used to formulate food blend that consists of cereal and soybeans in a ratio that is fit for consumption by growing children based on simple fermentation technology. They were able to solve the problem of malnutrition, growth retardation, infection and high mortality that is usually experienced by using foods that are not balanced for weaning in West Africa. Fermentation with *L. plantarum* is able to enhance the nutritive value of food by increasing the protein, mineral and vitamin content, reduce the pathogenic microbial load and make the food available in a form that is safe for consumption through the action of microbial activity (Buheloees, 2007).

1.1 Statement of Problem

Raffinose, ($C_{18}H_{32}O_{16}$), also known as melitose or melitriose contains many saccharide units which are not easily broken down into their simple sugars by many microorganisms including some LAB. Its hydrolysis is usually a 2- stage one because it contains alpha-1,4 and beta- 1,6 glycosidic bonds which makes it very difficult to be broken down. The 2-stage hydrolysis involves its breakdown first into sucrose and galactose, which in turn hydrolyses into glucose and fructose (Dictionary of Nutrition and Food Technology, 1990). This indirect breakdown of Raffinose often lead to late release

of glucose into the fermenting medium with a resultant effect on the efficiency of the different biochemical pathways required for breakdown.

The trisaccaharide raffinose cannot be absorbed through the wall of the small intestine into the blood stream, so in the absence of the enzyme that can digest this, raffinose present in the foods that are consumed remains uncleaved and passes into the colon. The operons of the enteric bacteria quickly switch over to raffinose metabolism and the resulting *in-vivo* fermentation produces copious amount of gas which is a mixture of hydrogen, carbon-dioxide and methane (Heyman, 2006; Townsend and Pitchford, 2012). This in turn may cause a range of abdominal discomfort, symptoms including cramps, bloating and flatulence in children. In addition, as with other unabsorbed sugars, such as raffinose, lactose, mannitol, sorbitol, its presence and its fermentation products raises the osmotic pressure of the colon thereby causing discomfort (Heyman, 2006; Townsend and Pitchford, 2012.).

1.2 Justification for the Work

Humans and other monogastric animals (pigs and poultry) do not possess the α-GAL enzyme required to break down RFOs (Gray *et al.*, 2012). This enzyme is produced by certain groups of enteric microorganisms and LAB which is able to break down this sugar into simple form. Raffinose, however, can be metabolized with limited success by certain groups of LAB especially *L. plantarum*. The ability of the indigenous *Lactobacillus* isolates to breakdown both *in vitro* and *in vivo* Raffinose and other members of the RFOs will go a long way in solving the problems enumerated above in the use of such components in foods of infant consumers.

1.3 Aims and Objectives of the Current work

Generally, this work is aimed at isolation, identification and characterizations of *L. plantarum* with capability to breakdown raffinose from various indigenously-fermented cereal gruels.

Specific objectives of the project include:

1. Characterization, including molecular, of *L. plantarum* from fermented gruels.
2. Optimization of the growth and nutrient utilization conditions of the isolates coupled with determination of the minimal basal requirements in raffinose.
3. Plasmid analysis of selected *L. plantarum* isolates and correlation with different physiological parameters

4. Fermentation studies and field application using cured and uncured *L. plantarum* isolates.
5. Production of good weaning blend for nutritional improvement with the action of *L. plantarum* in utilizing and metabolizing raffinose in the source.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The Group Lactic Acid Bacteria

2.1.1 General Characteristics

Lactic acid bacteria are a heterogeneous group of bacteria that are generally regarded as safe (GRAS). The use of these organisms in food products dates back to ancient time, and they are used mainly because of their contribution to flavour, aroma and increased shelf life of fermented products (Nes *et al*.,1996). Various members of this group are used commercially as starter cultures in the manufacture of foods including dairy products, fermented vegetable, fermented dough, alcoholic beverages, probiotics in animal feeds and meat products, lactic acid fermentation of sorghum and maize-based cereals used as infant weaning foods (Lorri and Svanberg, 1994).

The different genera include *Lactococcus, Leuconostoc, Pediococcus, Streptococcus* and *Lactobacillus.* These are important members of this group, which are commonly found in fermented foods that are commonly consumed. Other less well-known members of the group are *Sporolactobacillus, Carnobacterium, Enterococcus*, and *Bifidobacterium* (Jones, 1999). Some classifications also include *Oenococcus, Aerococcus, Teragenococcus, Vagococcus* and We*isella* (Grath and Sinderen, 2007). The various genera can be divided into species, sub species, varieties and strains. Each genus and species of Lactic acid bacteria has different characteristics based on their cellular morphology and biochemical activities used to identify them. Their metabolic activities as well as the various end-products of such metabolism are major distinguishable characteristics of LAB (Salminen and Wright, 1993). They are also defined on the basis of morphology, DNA base composition, and the type of fermentative metabolism and the pathway of such metabolism.

The Lactic acid bacteria are a broad group of Gram-positive, catalase negative, non-spore-forming cocci and rods, usually non-motile that utilize carbohydrates fermentatively and produce lactic acid as a major end product of such metabolism. (Aguirre and Collins, 1993). They vary in morphology from long, slender rods to short *Coccobacilli* which frequently form chains or clusters. All LAB grow anaerobically. Even though they can grow in the presence or absence of oxygen, they are aerotolerant organisms. They may utilize oxygen through enzyme flavoprotein-oxidase, while others are strictly anaerobic. Most strains have cell wall-bound proteinases, peptidases

mechanisms for active transport across the cell membrane (Dugas, 2004). They are oxidase, coagulase, urease and indole-negative and some reduce nitrate while others do not. Also, little or no proteolysis occurs in this group of microorganisms. They do not have the ability to hydrolyse casein and gelatin but they hydrolyse starch. They are readily killed by heat but some are able to grow at a temperature of 45^0C. The optimum temperature for growth is 30^0C. They are tolerant to acid with optimum pH range between 5.4 and 6.4 (Dugas, 2004).

The LAB are characterized as mesophilic and chemoheterotrophic. They are rarely non-pathogenic with the exception o*f Lactobacillus ca*se*i* which causes tooth decay known as dental caries. They are usually non-motile, non- sporulating organisms that produce lactic acid as a major product of fermentative metabolism. (Gilliand, 1990).They do not contain haemins i.e. cytochromes and catalase and so cannot synthesize them; also they cannot mediate the decomposition of hydrogen peroxide (Simpson and Taguchi, 1995). They lack the ability to synthesize porphyrins (components of respiratory chains) and therefore, cannot generate ATP by creation of a proton gradient. The Lactics can only obtain ATP by fermentation, usually of sugars and since they do not use oxygen in their energy production, they grow under anaerobic conditions (www.fsaa.u/ava/ca/lab).

Their nutritional requirement is a complex one; they are usually cultivated on media containing peptone, yeast extract or other digests of plant and animal materials supplemented with a fermentable carbohydrate to provide an energy source (APHA, 1992). The cells of LAB when grown on rich media produce the colonies that are relatively very small and never pigmented as a result of the absence of cytochromes. The growth has a chalky white appearance, with a shiny or glistering surface, which is a characteristic of LAB. Some species produce unusually large colonies when grown on sucrose-containing media as a result of the massive synthesis of extracellular polysaccharide either dextran or levan (APHA, 1992). Dugas (2004) in his work reported that they are fastidious organisms. They lack many biosynthesis capabilities and their complex nutritional requirement include needs for amino acids, vitamins, peptides, nucleotide bases, minerals, fatty acids, carbohydrates, purines and pyrimidines. They require accessory growth factors. No member of this group can grow on a purely salt medium without glucose and ammonium salts. They are therefore, mostly cultivated on complex media know as De man Rogosa and Sharpe (MRS) medium that contains relatively large amount of yeast extract, whey or even blood (www.waksmanfoundation.org/labs/mbl/lactic.html).

The largest genus in this order is *Lactobacillus* with almost 80 species. They are characterized by usually long, regular, non-sporing, Gram positive slender rods which frequently form chains (www. lactospore. com/back. htm). The genus is divided into 3 major groups based on their fermentation patterns. These are homofermenters, heterofermenters and the less well known heterofermentative species which produce DL-lactic acid, acetic acid and carbon (IV) oxide (www.lactospore.com/back.htm). They are usually non resistant to acidic conditions than the other members of this group.

Lactococcus are Gram positive cocci that appear ovoid depending on growth conditions and they are typically small in size. They grow in pairs or in short chains. They have complex nutritional requirement and are auxotrophic for a number of amino acids and vitamins (Batt, 1999). The optimum growth temperature of 30^0C and their failure to tolerate salt are diagnostic feature of this genus. They also differ in their ability to produce acid from sugars including lactose, mannitol and raffinose. The ability to ferment lactose is an important characteristics especially for those species used to produce dairy products (Salminen and Wright, 1993).

Leuconostoc are Gram positive cocci, which may be elongated or elliptical, arranged in pairs or chains. They lack catalase and cytochrome and carry out heterolactic fermentation with gas production by converting glucose to D-lactate and ethanol or acetic acid and CO_2 by means of phosphoketolase pathway (Funnel, 1999). They tolerate high sugar concentration so well that they grow in syrup and cause major problems in refineries. They have been found to produce the flavouring ingredients diacetyl and acetoin by the breakdown of citrate and have been used as starter cultures in dairy fermentations (www.en.wikipedia.org/wiki/lactic acid).

Streptococci occur in pairs or chains, they are usually non- motile and do not form endospores. They are chemoheterotrophs that ferment sugars to lactic acid and do not produce gas. The genus is made up of a wide variety of both pathogens and commensals which are Gram-positive bacteria that are found to inhabit man and animals (Jones, 1999).

Enterococci are spherical/ ovoid cells, which may appear in pairs or short chains. They are facultative anaerobes, which do not form spores. They ferment carbohydrate to lactate and carry out homolactic fermentation. It has been shown that they are at a high occurrence in traditional fermented seasoning foods. Its ability to tolerate high salt concentration, acid tests and low pH level has also been confirmed (Ohhira, 1999; http://en.wikipedia.org/wiki/lactic_acid).

Pediococcus are Gram positive, facultative anaerobe that is of great industrial importance. The formation of tetrads via cell division in two perpendicular directions in a single plane is a distinctive characteristic of *Pediococci* (Simpson and Taguchi, 1995).

The distribution of LAB is related to their high demand for nutrients and their type of energy generation. Most LAB obtain energy from the metabolism of sugar and related fermentable compounds and are hence restricted to habitats in which sugars are present (www.scielo.ve/scielo.php). They are therefore, found as normal flora of alimentary tract of man and animals. For example, *Lactobacillus casei* and *Lactobacillus brevis* have been found to be responsible for tooth decay in the mouth (www.en.wikpedia:org/wiki/lactic_acid).

2.1.2 Importance of LAB in Food Preservation and Contribution to Flavour

Morris (1991) reported the isolation of *L.acidophilus* and *L. fermentum*, which helps to lower the pH of the normal adult female genital tract. *Lactobacillus* species thus helps to prevent the growth of potential pathogens in the vagina, since their suppression by antibiotics can lead to the growth of *Candida albicans. L. salivarius* has also been isolated from the intestine and colon of animals (Levison and Jawetz, 1996). Friedman (2006) also reported the occurrence of *Lactobacillus plantarum* from intact and rotting plants. They are widely distributed in fermenting vegetables and animal products. *L.bulgaricus* and *L. helveticus* have also been isolated from agricultural products like milk, yoghurt, cheese and from fermented wine, cider and alcohol etc (www.dna22.com/projects/acid.html).

Lactic acid bacteria have also been isolated from several indigenous and African fermented foods such as *Ogi*, *Foofoo*, *Gari*, *kefir*, *kumiss*, *Tofu*, *Mawe* and drinks such as *Nunu*, Palm wine, *Agadagidi*, and *Burukutu* etc (Oyewole and Odunfa, 1990.) They are reported to contribute immensely to the flavour development, nutritional, organoleptic, and textural and improvement in vitamins of such foods. Some foods are also made edible while toxic substances such as cyanide from cassava are also removed as a result of fermentative activities of LAB and the enzymes produced by them (Oyewole and Odunfa 1990; 1998). Furthermore, LAB promotes sugar fermentation and modification of raw materials, which results in improvement in rheological and organoleptic properties. As a result of this, LAB have been used widely in the manufacture of dairy products including cheese, butter yoghurt, milk, fermented vegetable like pickles, olives,

sauerkraut, wine, cider and even sausage, dough, bread etc (Morris, 1991;Herrero *et al*., 1996).

Lactic acid bacteria are used in food industries for several reasons. Their growth lowers both the carbohydrate content and the pH of the foods that they ferment due to lactic acid production. This acidification process is one of the most desirable effects of their growth. The pH may drop to as low 4.0, low enough to inhibit the growth of most other microorganisms including most common human pathogens, thus conferring on these foods, prolonged shelf life (Dugas, 2004).

Lactococcus is one LAB that is used for the production of dairy fermentation products either as single strain starters or as part of a multiple-strain starter mix. The latter mixture consists of different strains form or multiple strains of different species (Batt, 1999). The *Lactococci* are usually used as starters in combination with other lactic acid bacteria starter cultures to ferment sugars to produce lactic acid which acidify the product as well as impart flavour development and preservation on such products (Salminen and Wright, 1993).

According to Smid *et al*. (2005) and Ammor *et al*. (2006), LAB is an essential part of the fermentation process in many food production industries. Fermentation with cultures containing LAB is able to produce healthy, safe, high quality and nutritious beneficial food products such as fermented milk, meat, vegetables, grains, cereals, legumes, meat, beverages etc. The organisms produce lactic acid which has a way of preserving such fermented foods and also improve the flavour, texture and nutritional compounds of such foods through the metabolic activities of LAB during fermentation. They are also present in different environments such as the alimentary canal of man, the mouth and the urinary tract exhibiting probiotic effect there. They have however been found to improve the microbial balance of the gastrointestinal tract of man and the other environment by their beneficial activities in man (Smid *et al.*, 2005 and Ammor *et al*., 2006).

2.2 *Lactobacillus plantarum*

2.2.1 Characteristics/Properties

2.2.1.1 Cultural and Morphological Characteristics of *L.plantarum*

It appears creamy, smooth and wet with raised elevation and does not produce pigments on MRS agar. It is a LAB with rounded ends. It occurs straight, singly, in pairs

or in short chains. It is motile and flagellation is ordinarily absent. It grows anaerobically in mediums containing peptone, yeast extract, carbon and nitrogen sources and salts (Sneath *et al.*, 1986).

Colonies are about 3mm, wide, raised, smooth, compact, creamy/white and occasionally light or dark yellow. Growth in broth results in an even heavy turbidity. Some strains ferment arabinose, raffinose, xylose, d-glucoside and d-mannoside. Ribose is fermented to one mole of lactic and one mole of acetic acid and other pentoses when fermented produce the same. Nitrates may or may not be reduced and ammonia is not produced from arginine. Optimal growth temperature range is usually between 30-35^{0}C and a pH of 5.5. Some essential amino acids such as niacin, thiamine, folic acid and vitamins B_{12} may or may not be needed for growth **(**Sneath *et al.*, 1986).

L. plantarum is a Gram-positive, aerotolerant bacterium that grows at 15^{0}C but not at 45^{0}C, and produces both isomers of lactic acid (D- and L-). This species and related *Lactobacilli* are unusual in that they can respire oxygen but have no respiratory chain or cytochromes. The consumed oxygen ultimately ends up as hydrogen peroxide. The peroxide, it is presumed, acts as a weapon to exclude competing bacteria from the food source. In place of the protective enzyme, superoxide dismutate present in almost all other oxygen-tolerant cells, this organism accumulates millimolar quantities of manganese polyphosphate. Manganese is also used by *L. plantarum* in a pseudo-catalase to lower reactive oxygen levels. Because the chemistry by which manganese complexes protect the cells from oxygen damage is subverted by iron, these cells contain virtually no iron atoms. Because of this *L. plantarum* cannot be used to produce active enzymes that require a heme- complex such as true catalases (Kleerebezem, 2003; http://www.ebi.ac.uk/2can/genomes/bacteria/Lactobacillus_plantarum.html).

Fructose1, 6-diphosphate aldolase is present and it also has hexose monophosphate activity. The growth of *L.plantarum* on gluconate is usually with carbon dioxide production. It ferments ribose to one mole of Lactic acid while nitrates are sometimes reduced, it does not produce ammonia from arginine (Prescott *et al.*, 2008). *Lactobacillus plantarum*, like many *Lactobacillus* species, can be cultured using MRS media.

2.2.1.2 Sources

L. plantarum is commonly found in many fermented food products including sauerkraut, pickles, brined olives, Korean *kimchi*, Nigerian *ogi*, sourdough, and other

fermented plant material, and also some cheeses, fermented sausages, and stockfish. The high level of this organism in food also makes it an ideal candidate for the development of probiotics. In the study of Frias and co-worker (2008), *L. plantarum* has been applied to reduce the allergenicity of soy flour. The result showed that, compared to other microbes, *L. plantarum*-fermented soy flour showed the highest reduction in IgE immunoreactivity (96–99%), depending upon the sensitivity of the plasma used. (http://www.ebi.ac.uk/2can/genomes/bacteria/Lactobacillus_plantarum.html).

2.2.1.3 Biochemical Characteristics of *L. plantarum*

2.2.1.3.1 Therapeutics

The ability of *L. plantarum* to survive in the human gastro-intestinal tract makes it a possible *in vivo* delivery vehicle for therapeutic compounds or proteins. The study of the peculiar characteristics of different strains of *L. plantarum* is fundamental for a better understanding of their potential in affecting the state of health, nutritional value, quality and stability of the foods that contain them (Taylor *et al.*, 2007, Frias *et al.*, 2008). *L. plantarum* strains are able to differentially influence the food quality when employed as starters. *L. plantarum*, like all probiotics, is a beneficial bacterium that can be used for improved health. *Lactobacillus* is the largest genus of the lactic acid bacteria group. *L. plantarum* is one of over 50 *Lactobacillus* species. It was first isolated from human saliva (Taylor *et al.*, 2007; Frias *et al.*, 2008).

Also, with promoting normal digestive health, *L. plantarum* has been shown to be an effective treatment for irritable bowel syndrome (IBS), Crohn's disease, and colitis. It has the ability to destroy pathogens and to preserve critical nutrients, vitamins, and antioxidants (Taylor *et al.*, 2007; Frias *et al.*, 2008). It has also shown the rare ability to produce, lysine, a beneficial amino acid. One of the most exciting uses of *L. plantarum* involves using it to deliver therapeutic compounds and proteins to the body. This area of research using the bacteria is new and ongoing (Taylor *et al.*, 2007; Frias *et al.*, 2008).

2.2.1.3.2 Antimicrobial Property

The ability of *L. plantarum* to produce antimicrobial substances helps them survive in the gastro-intestinal tract of humans. When a healthy colony of *L. plantarum* lives in the intestines, it prevents harmful bacteria from attaching to the mucosal lining and competes for the nutrients that the harmful bacteria need to survive. With no food and no room to live, the harmful bacteria pass harmlessly through the body. The antimicrobial substances produced have shown significant effect on Gram-positive and

Gram-negative bacteria. It has also shown some antifungal characteristics against some aflatoxin producing fungi (De Vuyst and Messens, 2002; Hugas *et al.*, 2003; Ogunbanwo *et al.*, 2003, Onilude *et al.*, 2004). One of the major probiotic properties for probiotic *L. plantarum* is its inhibitory effect on the growth of pathogenic bacteria. It is known to produce antimicrobial substances, e.g. plantaricin, that are active against certain pathogens. The production of antimicrobials is considered one of the major mechanisms through which probiotics function and consequently is also one of the principle criteria for strain selection when screening potential probiotics criteria for strain selection when screening potential probiotics (Wang *et al.*,2011).

2.2.1.3.3 Biochemistry

The entire genome has recently been sequenced, and promoter libraries have been developed for both conditional and constitutive gene expression, adding to the utility of *L. plantarum*. It is also commonly employed as the indicative organism in niacin bioassay experiments, as it is a niacin auxotroph (http://www.ebi.ac.uk/2can/genomes/bacteria/Lactobacillus_plantarum.html).

2.2.1.3.4 Proteolysis

Proteins are broken down into easily assimilable components. These activities of *L. Plantarum* and other *Lactobacilli* in the gastrointestinal tract make protein ingested by the host easily digestible, a property of great value in infant, convalescent and geriatric nutrition (Taylor *et al., 2007).* Lactic acid bacteria have the enzymes b-galactosidase, glycolases and lactic dehydrogenase (LDH) which produce lactic acid from lactose. Lactic acid, produced by *L. plantarum* is reported to have some physiological benefits such as:

a) Enhancing the digestibility of milk proteins by precipitating them in fine curd particles.
b) Improving the utilization of calcium, phosphorus and iron.
c) Stimulating the secretion of gastric juices
d) Accelerating the onward movement of stomach contents
e) Serving as a source of energy in the process of respiration.

The levels of optical isomeric forms of lactic acid produced depend upon the nature of the culture (Taylor *et al., 2007*).

Among the latest research on *L. plantarum* is that which has shown it to be highly effective in preventing soy-related allergies. In the work of Taylor *et al.* (2007) and Frias *et al.* (2008), soy seeds, flour, or meal was fermented using a variety of microorganisms. Fermented and unfermented soy products were introduced into blood plasma from people allergic to soybeans. *L. plantarum* enabled the greatest reduction in immunoreactivity to the soy products. In many cases, it was as much as a 99 percent reduction (Taylor *et al.*, 2007; Frias *et al.*, 2008). The process also increased the number of essential amino acids in the soy, along with new beneficial peptides, providing an additional health boost. Though a soy allergy only affects a small number of the population, that number is expected to grow as soy continues to become a more common ingredient in a wide range of food products (Taylor *et al.*, 2007; Frias *et al.*, 2008).

Products

These are some of the products that can be got from the various metabolic activities of *L. plantarum:*

2.2.1.3.5 Bacteriocin Production

Bacteriocins are proteins or protein complexes with bactericidal activities directed against species which are closely related to the producer bacterium. The inhibitory activity of *L. plantarum* towards putrefactive organisms is thought to be partially due to the production of bacteriocins. A number of bacteriocin are produced by *L. plantarum* some of them are Lactolin, Plantaricin B, Plantaricin N, Plantaricin, A, C, S, T and F (http://www.ebi.ac.uk/2can/genomes/bacteria/Lactobacillus_plantarum.html).

The antimicrobial ribosomal synthesized peptides produced by bacteria, including members of the LAB, are called bacteriocins. Such peptides are produced by many, if not all, *Lactobacillus* species and kill closely related microorganisms (Jack *et al.*, 1995). Due to their nature, they are inactivated by proteases in the gastrointestinal tract. Most of the LAB bacteriocins identified so far are thermo stable cationic molecules that have up to 60 amino acid residues and hydrophobic patches. Electrostatic interactions with negatively charged phosphate groups on target cell membranes are thought to contribute to the initial binding, forming pores and killing the cells after causing lethal damage and autolysin activation to digest the cellular wall (Gálvez et al., 1990).

The *L. plantarum* bacteriocins have many attractive characteristics that make them suitable candidates for use as food preservatives, such as:

- Protein nature, inactivation by proteolytic enzymes of gastrointestinal tract
- Non-toxic to laboratory animals tested and generally non-immunogenic
- Inactive against eukaryotic cells
- Generally thermo resistant (can maintain antimicrobial activity after pasteurization and sterilization)
- Broad bactericidal activity affecting most of the Gram-positive bacteria and some Gram-negative bacteria including various pathogens such as *L.monocytogenes*, *Bacillus cereus, S. aureus,* and *Salmonella spp.*
- Genetic determinants generally located in plasmid, which facilitates genetic manipulation to increase the variety of natural peptide analogues with desirable characteristics (Gálvez et al., 1990; Wang *et al.*, 2011).
-

2.2.1.3.6 Linamarase for linamarin activity

It produces the enzyme linamarase for linamarase activity which breakdown the linamarin in cassava (Giraud *et al.*, 1992). In this research by Giraud *et al.*(1992), *L. plantarum* strain was isolated from cassava, cultured on cellobiose- MRS medium showed a growth rate of 0.41 h^{-1}, a biomass yield of 0.22 g g^{-1}, and produced simultaneously an intracellular linamarase (76.4 U g^{-1} of biomass) and an extracellular amylase (36 U ml^{-1}(Giraud *et al.*, 1992).). The synthesis of both enzymes was repressed by glucose.

The use of such a strain as a cassava fermentation starter for *gray* production had the following influences: a change from a hetero-fermentative pattern observed in natural fermentation to a homofermentation, a lower final pH, a faster pH decline rate and a greater production of lactic acid (50 g kg^{-1} DM). However, this starter did not appear to play a significant role in cassava detoxification, since it was observed that the level of endogenous linamarase released during the grating of the roots was sufficient to permit the complete and rapid breakdown of linamarin (Giraud *et al.*, 1992).

2.2.1.3.7 Fermentation for the Production of Biofuel

L.plantarum has been implicated in the fermentation of cassava for ethanol production. Biofuels are fuels made from recently living organisms. They can be divided into three categories; those that are made largely from edible sugars and starches, those that are made from non-edible plant materials. Others are made from

algae and other microbes (Siqing, 2006; Wang *et al*., 2011). Biofuels are renewable, meaning their sources can be regrown. Advanced biofuels can offer environmental benefits such as lower carbon emissions and lower sulphur compared with first-generation biofuels and conventional petroleum-based fuels (Siqing, 2006).

Gram-positive bacteria have been explored to convert lignocellulosic biomass to biofuel and bioproducts. In the work of Siqing, 2006, the goal of the work was to create genetically engineered lactic acid bacteria (LAB) strains that could convert agricultural biomass into ethanol and other value-added products (Siqing, 2006; www.usda.gov). This was done either by introducing ethanol production pathway genes or inactivating pathways genes that lead to production of undesired byproducts (Siqing, 2006; Wang *et al.*, 2011; www.usda.gov).

The widely studied species, *L. plantarum* is now considered a model for genetic manipulations of LAB. In this study, *L. plantarum* TF103 strain, in which two of the chromosomal L-*ldh* and D-*ldh* genes are inactivated, was used to introduce additional mutations on the chromosome to eliminate undesired fermentation products. This method should facilitate research in targeted inactivation of other genes in LAB (Smid *et al*., 2005; Siqing, 2006;www.usda.gov).

2.2.1.3.8 Food Preservative and flavours

Lactobacillus plantarum has been used for the preservation of food for increased shelf life and flavours to get the desired aroma in food (Daeshel, 2004). *L. plantarum* is one of the lactic acid producing bacteria that have been used for centuries for the preservation of human food. It is a simple, safe method that is still used in many undeveloped countries. In addition, scientists conducting the study stated that lactic acid fermentation, such as that used with *L. plantarum*, is the safest way to preserve food. It is one of the most versatile probiotics, *L. plantarum* is found in plant material and the gastrointestinal tract of animals, including humans (Smid *et al.*, 2005 and Ammor *et al*., 2006).

It is used in the fermentation of foods like sauerkraut, kimchi, pickles, and sourdough bread. It has been used for this purpose for hundreds of years, and is a healthier option in food preservation (Taylor *et al.*, 2007; Frias *et al.*, 2008). For example, *Iru*, *Ogiri* produced in South West, Nigeria, it is used as a result of contribution to flavour of the food (Odunfa, 1985). Also, curry paste is a popular wet blend of spices and herbs in Malaysia and most of Asia which is consumed as chicken, meat or fish gravy and as a

vegetable seasoning. Curry paste is primarily known as an important ingredient in Thai cuisine and it can also be a generic commercial product which substitutes curry powders or spice blends used in other cuisines (Abolhassani *et al.*, 2009)**.**

L. plantarum is able to increase the shelf life of the food product in which it is found and it can be considered as the main point of interest and addition of flavour. The flavour profile of volatile compounds of curry paste mentioned above and other fermented flavours indicated some production of organic compounds. This slight increase in aromatic compounds may result in enhanced consumer acceptability of fermented foods and locally made food flavours (Abolhassani *et al.*, 2009; Amankwah *et al.*, 2012,).

2.2.2 Functional properties /importance of *L. plantarum*

The genus *Lactobacillus* encompasses an important group of microorganisms that are used in the fermentation and production of a variety of dairy products, vegetables, cereals, legumes, bakery products, meat and silage (Signe *et al*., 1999).These bacteria produce lactic acid and characteristic flavour in food. Some strains have been used as intestinal flora controllers (Salminen *et al*., 1993; Smid *et al.*, 2005).

One of the most widespread strains used in food technology is *Lactobacillus plantarum.* This organism has a homofermentative ability and produce lactic acid as an end product of metabolism; and depending on the growth rate and pH level, of substrate in oxygen medium, it can also produce acetic acid and acetoin (Barneto *et al*., 1996).

2.2.2.1 Use as Probiotics

Fermented foods are associated with 'good bacteria' referred to as **Probiotics.** *Lactobacillus plantarum* has however been named as one of those good, useful and safe bacteria (Patricia *et al*., 2002; Helland *et al*., 2004; Smid *et al.*, 2005 and Ammor *et al*., 2006).

Probiotics, as defined in a FAO/WHO (2002) report, are 'live microorganisms which when administered in adequate amounts confer a health benefit on the host'. Probiotics are beneficial bacteria in that they favourably alter the intestinal microflora balance such as reconstruction of normal intestinal microflora after disorders caused by diarrhoea, antibiotic therapy and radiotherapy. It inhibits the growth of harmful bacteria, promote good digestion, boost immune function and increase resistance to infection (Patricia *et al*., 2002; Helland *et al*., 2004).

Other physiological benefits of probiotics include removal of carcinogens, lowering of cholesterol, immune-stimulating and allergy lowering effect, synthesis and enhancing the bioavailability of nutrients (Grajek *et al*., 2005; Parvez *et al*., 2006). People with flourishing intestinal colonies of beneficial bacteria are better equipped to fight the growth of disease causing bacteria (Holzapfel and Schillinger, 1992; Helland *et al*., 2004).

Fermentation is one of the oldest technologies used for food preservation and *Lactobacillus plantarum* is one of such organisms that are used. The organism produce organic acids and probable bacteriocins in the fermentation process that results in the prevention and growth of microorganisms hence increasing the shelf life of fermented products (Chen and Hoover, 2003;Corsetti, 2004). This is a very valuable attribute especially in rural areas where advanced food preservation technologies such as refrigeration are not affordable, also people have began to appreciate more of naturally preserved food than chemically preserved foods (Rolle andSatin, 2000).

Several studies have however, been carried out that shows *L.plantarum* as a potential probiotics. For example, the study of Kalui *et al.* (2008) has shown that some *L. plantarum* strains isolated from '*ikii'* a fermented food have potential probiotic attributes. Some of the assayed strains showed antimicrobial activity which may have been beyond the effect of organic acids, and had bacteriocin production attributes. Some assayed *L. plantarum* strains showed tolerance to low pH (2) and growth in 0.3% bile conditions hence are able to survive and grow in physiological conditions of the Gastro Intestinal Tract (GIT) (Kalui *et al.*, 2008). The assayed strains showed safety with regard to haemolytic and gelatinase activity. They were able to produce Exopolysaccharides and some were able to utilise Fructooligosaccharide, all of which are characteristics attributed to probiotics.

Also, in the work of Kaban *et al.* (2009) on dry fermented sausage called "Sucuk" from China, *L.plantarum* was shown as possessing antibacterial activities. Enterobacteriaceae, which are sensitive to acid and water activity, were progressively eliminated from the ripened '*sucuk'*. However, it was reported that fast growth of lactic acid bacteria, with the consequence of deep acidification of the substrate, could result in inhibition toward these microorganisms that exhibit pathogenic or disease causing attributes (Rantisiou and Cocolin, 2006). This is also confirmed by the different works of Onilude *et al.* (1993), Nout (1991), Reddy and Pierson (1994); Tucker and Woods (1995), Ogunbanwo *et al.* (2003) and Wakil and Onilude (2009). *L.plantarum* has also

been implicated in its major functions as it contributes to colour formation, stabilization and aroma development by means of their catalase, nitrate and nitrite reductase activities and implication in lipid metabolism (Lucke 1998; Toldra *et al.*, 2001).

L.plantarum ferments raffinose and lactose efficiently and possesses alpha-galactosidase, beta-galactosidase and beta-phosphogalactosidase activities. It also shows high antimicrobial activities on various test organisms tested (Mensah *et al.*, 1990; Hugas *et al.*, 1993; Leisner *et al.*, 1996; Nes *et al.*, 1996; Klein *et al.*, 1998). All the strains had a high content of extra chromosomal DNA of different functions. A recombinant plasmid from *L. plantarum* has been constructed and may be used to develop a shuttle vector for different species of microorganism (Mayo *et al.*, 1998; Wang *et al.*, 2011).

These special and useful attributes of *L.plantarum* was conferred on it by the possession and presence of *plasmids* which is an extra-chromosomal DNA molecule that confers these attributes on such organisms known as Probiotics. Plasmids are essential for the bacteria in that they may give it a selective advantage. Some plasmids determine the production of proteins that can kill other bacteria; others make bacteria resistant to antibiotics. Plasmids are extremely valuable tools in the fields of molecular biology and genetics, specifically in the area of genetic engineering (Bruce *et al.*, 2002).

It is imperative therefore that research and technology is now focusing a lot of attention on fermentation technologies and their products with an aim of tapping into the possible associated health benefits.

2.2.2.2 Production of Cheese, Yoghurt and Wine

L. plantarum is used as starter culture in food industry for the production of cheese, yoghurt and other fermented foods and beverages (Monica *et al.*, 1999). Proteolytic system of *L. plantarum* has been mainly studied with reference to cheese technology, as it plays an important role in the texture and flavour development of cheese (Wilkinson *et al.*, 1995). In order to produce cheese, yoghurt and wine of consistent quality, starter cultures (similar to those used in the dairy industry) have been recommended.

Not only do starter cultures ensure consistency between batches, they speed up the fermentation process as there is no time lag while the relevant microflora colonise the sample (Wilkinson *et al.*, 1995; Amankwah *et al.*, 2012,). Because the starter cultures used are acidic, they also inhibit the growth of undesirable micro-organisms. It is possible

to add starters traditionally used for milk fermentation, such as *L. plantarum or Streptococcus lactis*, without adverse effect on the final quality of the product.

Because these organisms only survive for a short time (long enough to initiate the acidification process) in the fermenting medium, they do not disturb the natural sequence of micro-organisms (Wilkinson *et al.*, 1995). On the other hand, if *L. plantarum* is added as starter in the early stages, it gives a good flavour to the final product, but alters the sequence of subsequent bacterial growth and results in a product that is completely fermented. This does not disturb the balance between acetic and lactic acids and the fermentation reaches completion. If the right starter is not chosen, the final product is not completely fermented and the resulting product is bitter and more susceptible to spoilage by yeasts (Monica *et al.*, 1999; Amankwah *et al.*, 2012,).

2.2.2.3 Production of Organic acids and Aromatic compounds

L. plantarum has been implicated in the production of Organic acids and Aromatic compounds such as Lactic acid, Acetic Acids, etc (Monica *et al.*, 1999).The production of volatile compounds by the probiotic strain, *L. plantarum* in cereal-based media (oat, wheat. barley and malt) was investigated in the work of Salmeron *et al.* (2009) and co-workers. Sixty compounds, including fatty acids and their esters, amides, alcohols, aldehydes, aromatic hydrocarbons, furans, ketones, peroxides and pyrans, were identified (Salmeron *et al.*, 2009).

L. plantarum significantly changed the aroma profile of the four cereal broths. The most abundant volatiles detected in oat, wheat, barley and malt were oleic acid, linoleic acid, acetic acid, and 5-hydroxymethylfurfural, respectively. Analysis of these products confirmed the heterofermentative pathway of *L. plantarum*. Maillard compounds were not detected during sterilization and fermentation. The results obtained could contribute to the development of new non-dairy probiotic formulations (Smid *et al.*, 2005; Ammor *et al.*, 2006; Salmeron *et al.*, 2009).

2.2.2.4 Proteinase Activity

L. plantarum has shown proteinase activity in some medium of growth on a large scale in the industry to get the desired end product (Ghosh and Marathe, 2009).*L. plantarum* is used in the dairy industry with other lactic acid bacteria to produce fermented milk, sour milk and yoghurt (Ghosh and Marathe, 2009). The demand for processed dairy foods has increased considerably with growing urbanization, especially for different cheese varieties and low-lactose milk. Proteinase also helps to reduce the

allergic properties of milk and milk products for infants which can lead to a severe nutritional problem of protein-energy deficiency (Monica *et al.,* 1999; Amankwah *et al.,* 2012,

L. plantarum are important organisms used in several lactic acid fermentations. If is cultured in milk, it performs activities like breaking down of lactose to simple sugars, proteolysis, lipolysis and lactic acid production. *L.plantarum* depends on proteolytic system for degradation of protein – casein in milk from which they get their complex nutrient requirements for growth (Smid *et al.,* 2005; Ammor *et al*., 2006; Ghosh and Marathe, 2009). This complex proteolytic system consists of proteinases, peptidases and amino and peptide carriers. Yogurt fermented milk having increased nutritive value, is more palatable, easily digestible and assimilable than milk. It has been observed that many people are sensitive to milk and cannot digest it easily. In developing nations, this is an important cause for concern as such sensitive people suffers from protein-energy deficiency, more so if they are from the economically weaker section of the society (Smid *et al.,* 2005 ;Ammor *et al*., 2006;Ghosh and Marathe, 2009; Amankwah *et al.,* 2012)

2.2.2.5 Gene Manipulation

In various biotechnological processes, the gene of *L. plantarum* can be manipulated for genetic purposes and strain improvement. Genetically manipulated combinants of L. *plantarum* can proliferate and compete with epiphytic lactic acid bacteria in silage. Ensiling is a major method of forage conservation in temperate countries and is used to provide a feed of high nutritive value for ruminants (Sharp *et al*., 1992; Nagai *et al.,* 2012).

Silage is produced by the anaerobic fermentation of sugars in the forage by the indigenous microflora. The course of the silage fermentation is the growth and persistence of two genetically manipulated forms of *L. plantarum*. Both recombinants contained pSA3, a shuttle vector for gram-positive organisms that encodes erythromycin resistance (Sharp *et al*., 1992; Nagai *et al.*, 2012).

In one of the recombinants, pSA3 was integrated onto the chromosome, whereas in the other, a pSA3 derivative designated pM25, which contains a *Clostridium thermocelum* cellulase gene cloned into pSA3, was maintained as an extra chromosomal element. This extra chromosomal element is a plasmid. Since strains of *L. plantarum* are

frequently used as silage additives, there is considerable potential in genetically manipulating this bacterium to enhance its fermentation (Sharp *et al*., 1992).

2.2.2.6 Silage

L. plantarum is the most common bacterium used in silage inoculants. During the anaerobic conditions of ensilage, these organisms quickly dominate the microbial population and, within 48 hours, they begin to produce lactic and acetic acids via the Embden–Meyerhof Pathway, further diminishing their competition. Under these conditions, *L. plantarum* strains producing high levels of heterologous proteins have been found to remain highly competitive. This quality could allow this species to be utilized as an effective biological pre-treatment for lignocellulosic-biomass (Sharp *et al*., 1992; Hymowitz, 2012; http://www.pubmedcentral.nih.gov/articlerender.fcgi?tool=pmcentrez&artid=14946).

To better control the ensilage process, homofermentative lactic acid bacteria are being increasingly used as silage inoculants. Since strains of *L. plantarum* are frequently used as silage additives, a limiting factor in the fermentation of grass silages is the availability of soluble sugars as a substrate for a rapid fermentation (Sharp *et al*., 1992).

A recent development to alleviate this problem has been the addition of commercially produced cellulases and hemicellulases during ensilage to release soluble sugars from the degradable fiber components. The work also describes a method for detecting genetically manipulated *L. plantarum* in silage by using a dual antibiotic selection system (Sharp *et al*., 1992; Hymowitz, 2012).

The efficiency of this approach was confirmed by Southern hybridization. Unfortunately, this technique only indicates the presence of specific DNA sequences having homology with the probes used. Southern hybridization does, however, provide an indication of the size of the DNA fragment showing homology with the probe (Sharp *et al*., 1992; Hymowitz, 2012).

2.3 LAB Metabolism

2.3.1 Lactic Acid Fermentations

Lactic acid fermentation is the simplest type of fermentation. In essence, it is a redox reaction. In anaerobic conditions, the cell's primary mechanism of ATP production is glycolysis (Klein *et al*., 2004). Glycolysis reduces transfers electrons to NAD^+, forming NADH. However, there is only a limited supply of NAD^+ available in a cell. For

glycolysis to continue, NADH must be oxidized, i.e. have electrons taken away to regenerate the NAD^+. This is usually done through an electron transport chain in a process called oxidative phosphorylation; however, this mechanism is not available without oxygen (Klein *et al.*, 2004).

Instead, the NADH donates its extra electrons to the pyruvate molecules formed during glycolysis. Since the NADH has lost electrons, NAD^+ regenerates and is again available for glycolysis. Lactic acid, for which this process is named, is formed by the reduction of pyruvate (Madigan *et al.*, 1996).

2.3.1.1 Health Benefits of Lactic Acid Fermentation

Fermented food, enjoyed across the globe, conveys health benefits through lactic acid fermentation. The fermentation process can transform the flavour of food from the plain and mundane to a mouth-puckering sourness enlivened by colonies of beneficial bacteria and enhanced micronutrients (Taylor *et al.*, 2007; Frias *et al.*, 2008). While fermented food like yogurt, sauerkraut and kefir are well-known, many other lesser-known foods also benefit from the lactic acid fermentation process. Indeed, virtually every food with a complex or simple sugar content can be successfully fermented.

Born of both necessity and practicality, lactic acid fermentation proved to be not only an efficient method of preserving food, but also a critical one. Indeed, fermented foods like sauerkraut, cheese, wine, kvass, soured grain porridge and breads often sustained tribes and villages during harsh winters when fresh food simply wasn't available let alone plentiful (Hymowitz, 2012; Gray *et al.*,2012; *http://www.heaveninthehome.com*).

In many societies where yogurt has been heralded as a health food since the 19th century, fermented foods has gained a reputation for its beneficial effects on immunity, intestinal health and general well-being. Modern researchers are just beginning to understand what the sages of old were tuned in to. Fermented foods convey clear and calculable health benefits to the human diet. Lactic acid fermentation in and of itself enhances the micronutrient profile of several foods. For example, milk that undergoes lactic acid fermentation either in the wild as in the case of clabbered milk or inoculated by a starter culture as in the case of yoghurt, *piima, matson*i and other fermented dairy products conveys more vitamins to the eater in comparison to raw milk and, particularly, pasteurized and ultra-high-temperature pasteurized milk. Fermented dairy products consistently reveal an increased level of folic acid which is critical to producing healthy

babies as well as pyroxidine, B vitamins; riboflavin and biotin depending on the strains of bacteria present (Gray *et al.*,2012;*http://www.heaveninthehome.com/*).

The increase in the micronutrient profiles of fermented foods is not just limited to yoghurt, bonny clabber and kefir. Vegetables, fruits, legumes and grains subjected to lactic acid fermentation also see increases in both their macro- and micronutrient profiles. The bioavailability of amino acids particularly lysine with its antiviral effects and methionine - increases with lactic acid fermentation (Odunfa, 1985).For grains, sprouting prior to souring can increase the availability of protein even further. Vegetables that have undergone lactic acid fermentation as in the case of sauerkraut and kimchi often see an increase in the activity of vitamin C and vitamin A (Wang *et al*., 2011).

While lactic acid fermentation does not usually increase the level of minerals present in fermented foods unless unusual circumstances are present (as in fermenting food in a metal or earthen container), it does decrease the activity of phytic acid content naturally present in grains (Taylor *et al.*, 2007; Frias *et al.*, 2008). Phytic acid is an antinutrient that binds up minerals preventing full absorption of minerals in the gut. Since souring grains reduces the phytic acid content, the lactic acid fermentation process actually enables our body to absorb more minerals from the grain than you would be able to otherwise absorb (Taylor *et al.*, 2007; Frias *et al.*, 2008; Gray *et al.*, 2012).

It is then possible to eliminate modern sweeteners from foods that are consumed by children and adult, and incorporate fermented foods into diet and take advantage of all the health benefits that lactic acid fermentation offers. In this way, lactic acid fermentation is able to improve our health (*http://www.heaveninthehome.com/).*

2.3.1.2 Fermentation: General Consideration

Fermentation is an alternate metabolic pathway that allows glucose to be partially metabolized when there is no final electron acceptor available to complete the electron transport chain. Fermentation consists of the first stage of cellular respiration- glycolysis and then the reduction of the electron carrier NAD^+. The term glycolysis describes exactly what is occurring during this metabolic step- the lysis, or breaking down, of the sugar glucose. During glycolysis, the glucose molecule is cut in half, a catabolic reaction that releases energy, resulting in two ATP, two molecules of the electron carrier NADH, and two molecules of pyruvic acid (Bauman,2007; Tortora *et al*.,2010) . It is the process by which the electrons and hydrogen ions from the NADH produced by glycolysis are donated to another organic molecule. It is a metabolic process

whereby electrons released from nutrients are ultimately transferred to molecules obtained from the breakdown of those same nutrients (Dickinson, 1999).

Fermentation is also the process of extracting energy from the oxidation of organic compounds, such as carbohydrate, and using an endogenous electron acceptor, which is usually an organic compound. Fermentation is important in anaerobic conditions when there is no oxidative phosphorylation to maintain the production of ATP (Adenosine triphosphate) by glycolysis. During fermentation, pyruvate is metabolised to various different compounds (Dickinson, 1999; Klein *et al*., 2004).

Fermentation in food processing typically is the conversion of carbohydrates to alcohols and carbon dioxide or organic acids using yeasts, bacteria, or a combination thereof, under anaerobic conditions. A more restricted definition of fermentation is the chemical conversion of sugars into ethanol. Fermentation usually implies that the action of microorganisms is desirable, and the process is used to produce alcoholic beverages such as wine, beer, and cider. Fermentation is also employed in the leavening of bread, and for preservation techniques to create lactic acid in sour foods such as sauerkraut, dry sausages, cheese and yoghurt, vinegar and pickles (Steinkraus, 1995).

According to FAO (2007), the primary benefit of fermentation is the conversion of sugars and other carbohydrate e.g., converting juice into wine, grains into beer, carbohydrates into carbon dioxide to leaven bread, and sugars in vegetables into preservative organic acids. In Africa**,** fermented foods and beverages include millet porridge, *garri*, hibiscus seed, hot pepper sauce, *foofoo*, oil seed, *ogi*, *ogiri* and palmwine,*agadagidi* and *burukutu*. In Asia - *keffir, Tofu, sake, soju* and soy sauce, while in America, it includes Pickles, oil seed, chocolate, vanilla, saukraut and in Europe - elderberry wine, salami, skyr and cultured milk products such as kefir and quark. Fermented foods and beverages are thus consumed worldwide (FAO, 2007).

Fermentation can be divided into 2 types: Homolactic and Heterolactic fermentation.

2.3.1.3 Homolactic Fermentation

Homolactic fermentation is the production of lactic acid from pyruvate. In homolactic acid fermentation, both molecules of pyruvate are converted to lactate. It is unique because it is one of the only respiration processes that do not produce gas as a by-product. Homolactic fermentation breaks down the pyruvate into lactate. It occurs in the

muscles of animals when they need energy faster than the blood can supply oxygen. It also occurs in some kinds of bacteria (such as *Lactobacillus sp*) and some fungi. It is this type of bacteria that converts lactose into lactic acid in yoghurt, giving it its sour taste (Dickinson, 1999; Klein *et al*., 2004).

In homolactic fermentation, one molecule of glucose is converted to two molecules of lactic acid:

$$C_6H_{12}O_6 \rightarrow 2\ CH_3CHOHCOOH$$

This utilizes the Embden-Meyerhof Parnas pathway. It is the most common pathway for glucose degradation to pyruvate in stage two of anaerobic respiration. It is found in all major groups of microorganisms and functions in the presence or absence of oxygen. It also occurs in the cytoplasm matrix of prokaryotes and eukaryotes. It is divided into two parts; the initial 6-carbon phase, in this, energy is consumed as glucose in phosphorylase twice and is converted to fructose 1, 6-bisphosphate.

Fructose 1, 6-bisphosphate aldolase catalyzes the cleavage of fructose 1, 6-bis phosphate into two halves, each with a phosphate group. One of the products, dihydroxy acetone phosphate, is immediately converted to glyceraldehyde 3-phosphate. This yields 2 molecules of glyceraldehyde 3-phosphate, which is then converted to pyruvate in a five step process. Dihydroxyacetone phosphate can be easily changed to glyceraldehyde-3-phosphate, both halves of fructose 1, 6-bi-phosphate are used in the three carbon phase.

First, glyceraldehyde 3-phosphate is oxidized with NAD^+ as the electron acceptor to form NADH and a phosphate is simultaneously incorporated to give a high energy molecule called 1,3-bisphosphate. The high energy phosphate on carbon one is subsequently donated to ADP to produce ATP. This is called substrate level phosphorylation (Prescott *et al.*, 2008).

2.3.1.4 Heterolactic Fermentation

In heterolactic fermentation, the reaction proceeds as follows, with one molecule of glucose being converted to one molecule of lactic acid, one molecule of ethanol, and one molecule of carbon dioxide:

$$C_6H_{12}O_6 \rightarrow CH_3CHOHCOOH + C_2H_5OH + CO_2$$

Before lactic acid fermentation can occur, one molecule of glucose must be split into two molecules of pyruvate. This process is called glycolysis (Voet and Voet, 1995). Heterolactic fermentation is the production of lactic acid as well as other acids and alcohols. In heterolactic acid fermentation, one molecule of pyruvate is converted to

lactate; the other is converted to ethanol and carbon dioxide. The lactic acid bacteria (LAB) can be classified as homofermentative, where the end-product is mostly lactate, or heterofermentative, where some lactate is further metabolized and results in carbon dioxide, acetate, or other metabolic products (Voet and Voet, 1995).

This utilizes the pentose phosphate pathway. It begins with the oxidation of glucose 6-phosphate to 6-phospho gluconate followed by the oxidation of 6-phospho gluconate to the pentose sugar ribulose 5-phosphate and CO_2.NADPH is produced during the oxidations. Ribulose 5-phosphate is then converted to a mixture of three through seven-carbon sugar phosphates. Two enzymes play a central role in these transformations. First, transketolase catalyzes the transfers of a three-carbon group from sedoheptulase 7-phosphate to glyceraldehyde 3-phosphate. The overall result is that three glucose 6-phosphate is converted to two fructose 6-phosphate, glyceraldehydes 3-phosphate and three CO_2molecules.

3 glucose 6-phosphate + 6NAD + $3H_20$ + 2 fructose 6- phosphate + glyceraldehydes 3-phosphate + $3CO_2$ + 6 NADPH + 6H

These intermediates are used in two ways; the fructose 6-phosphate can be changed back to glucose 6-phosphate while glyceraldehydes 3-phosphate is converted to pyruvate by enzymes of the Embden-Meyerhof pathway. Alternatively, 2 glyceraldehyde 3-phosphate may combine to form fructose 1, 6-biphosphate, which is eventually converted back into glucose 6-phosphate. This results in the complete degradation of glucose 6-phosphate to CO_2 and the production of a great deal of NADPH (Prescott *et al.*, 2008).

2.3.1.5 Importance of Lactic Fermentation

Fermentation is the process of biological conversion of organic substances by microorganisms and enzymes of microbial, plant or animal origin to give desirable fermented foods with great biochemical changes and modification of food quality**.** It is the oldest form of food preservation that is available locally (FAO/UN, 1994). Fermentation is globally applied in the preservation of a range of raw agricultural materials (cereals, legumes, roots, tubers, fruits and vegetables, milk, meat, fish etc) and the process is mostly the outcome of lactic acid bacteria- yeast interaction (Sanni *et al.*, 1995). Odunfa (1985) stated that the most important substrates for fermented foods in tropical Africa are cereal grains. These include sorghum, maize and millet.

Steinkraus (1995) pointed out that the traditional fermentation of foods serves several functions such as enrichment of food substrate biologically with proteins, essential amino acids, essential fatty acids and vitamins; this improves digestibility and acceptability of foods, detoxification of toxic substances in foods, preservation of substantial amounts of food through production of antibacterial compounds such as lactic acid and acetic acid, a decrease in time and fuel requirement during cooking and enrichment of the diet through the development of flavours, aroma, taste, palatability and texture of such foods.

The content and quality of cereal proteins is also improved by fermentation. Natural nutritive value and available lysine are also improved (Cahvan *et al.*, 1988). Bacterial and yeast fermentations involving proteolytic activity are expected to increase the biological availability of essential amino acids and degrade carbohydrates. Different changes also occur in the vitamin content especially the B group vitamins based on the fermentation process and the raw materials used for fermentation (Cahvan *et al.,* 1989; Smid *et al.,* 2005; Ammor *et al*., 2006).

Reddy and Pierson (1994) reviewed the effect of fermentation on anti-nutritional and toxic components in plant foods. Fermentation lowers the flatus producing carbohydrate of soybean, trypsin inhibitor and phytates. Nout (1991) also noted that fungal and lactic acid fermentations have been found to reduce aflatoxin B_1 by opening of the lactose ring which results in complete detoxification.

There are two main hexose fermentation pathways that are used to classify LAB genera. Under conditions of excess glucose and limited oxygen, homolactic LAB catabolize one mole of glucose in the Embden-Meyerhof-Parnaz (EMP) pathway to yield two moles of pyruvate. Intracellular redox balance is maintained through the oxidation of NADH, concomitant with pyruvate reduction to lactic acid. This process yields two moles of ATP per glucose consumed (Salminen *et al*., 2004, Madigan *et al*., 2004).

Heterofermentative LAB use the Pentose phosphate pathway, alternatively referred to as the pentose phosphoketolase pathway. One mole Glucose-6-phosphate is initially dehydrogenated to 6-phosphogluconate and subsequently decarboxylated to yield one mole of CO_2 (Madigan *et al.*, 2004). The resulting pentose-5-phosphate is cleaved into one mole glyceraldehyde phosphate (GAP) and one mole acetyl phosphate. GAP is further metabolized to lactate as in homofermentation, with the acetyl phosphate reduced to ethanol via Acetyl-CoA and acetaldehyde intermediates. In theory, end-products

(including ATP) are produced in equimolar quantities from the catabolism of one mole of glucose (Salminen *et al.*, 2004, Madigan *et al.*, 2004).

2.4 Raffinose and Raffinose Metabolism

2.4.1 The Oligosaccharide Raffinose

Raffinose is a trisaccharide composed of galactose, fructose, and glucose. It can be found in beans, cabbage, Brussels sprouts, broccoli, asparagus, other vegetables, and whole grains. It can also be found in honey and brown sugar. Raffinose can be hydrolyzed to D-galactose and sucrose by the enzyme alpha-galactosidase and beta-galactosidase (α andβ -GAL), enzymes not found in the human digestive tract. Alpha-GAL also hydrolyzes other α-galactosides such as stachyose, verbascose, and galactinol, if present. The enzyme does not cleave β-linked galactose, as in lactose (Storey *et al.*, 1998; Townsend and Pitchford, 2012).

The raffinose family of oligosaccharide (RFOs) is alpha-galactosyl derivatives of sucrose, and the most common are the trisaccharide raffinose, the tetrasaccharide stachyose, and the pentasaccharide verbascose. RFOs are almost ubiquitous in the plant kingdom, being found in a large variety of seeds from many different families, and they rank second only to sucrose in abundance as soluble carbohydrates (Storey *et al*, 1998; Townsend and Pitchford, 2012). It is a dextrorotatory trisaccharide, occurring in cotton seed and in the molasses of beetroot, composed of d-galactose, d-glucose, and d-fructose and formed by transfer of d-galactose from UDP-d-galactose (http.www.righthealth.com/corp/doingright).

Sugars of the raffinose series consist of α1, 6-linked chains of d-galactose attached to the 6-glucosyl position of sucrose (Peterbauer *et al.*, 2002; Gray *et al.*, 2012). They are synthesized in leaves, roots, and tubers of a range of plant species. In seeds of higher plants, they are of almost ubiquitous occurrence. In some crop species, raffinose oligosaccharides comprise up to 16% of seed dry matter. Aside from a role as storage and transport carbohydrates, other functions of these oligosaccharides remain elusive. In the specialized, desiccation-tolerant seeds of higher plants, raffinose and its higher homologues may play a role as protective agents during maturation drying. Furthermore, correlative and experimental data suggest they may act as cryoprotectants in frost-hardy plants (Peterbauer *et al.*, 2002; Gray *et al.*, 2012; Townsend and Pitchford, 2012).

Humans and other monogastric animals (pigs and poultry) do not possess the α-GAL enzyme required to break down RFOs. These oligosaccharides however pass undigested through the stomach and upper intestine. In the lower intestine, they are fermented by gas-producing bacteria which do possess the α-GAL enzyme and make carbon dioxide, methane, and hydrogen leading to the reduction in the flatulence commonly associated with eating beans and other vegetables (Thompson, 1998).

2.4.1 The Structure and Molecular Formula of Raffinose

The oligosaccharide raffinose has a molecular formula C_{18} H_{32} O_{16} and a molar mass of 504.42g/mol. It is also being called by some other names such as Gossypols, melitose, melitriose, α-D-Galactosylsucrose. It is a pentahydrate with IUPAC name (*2R,3R,4S,5S,6R*)-2-[(*2S,3S,4S,5R*)-3,4-Dihydroxy-2,5-bis(hydroxymethyl) oxolan-2-yl]oxy-6-[[(*2S,3R,4S,5R,6R*)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-oxymethyl]oxane-3,4,5-triol.

Raffinose

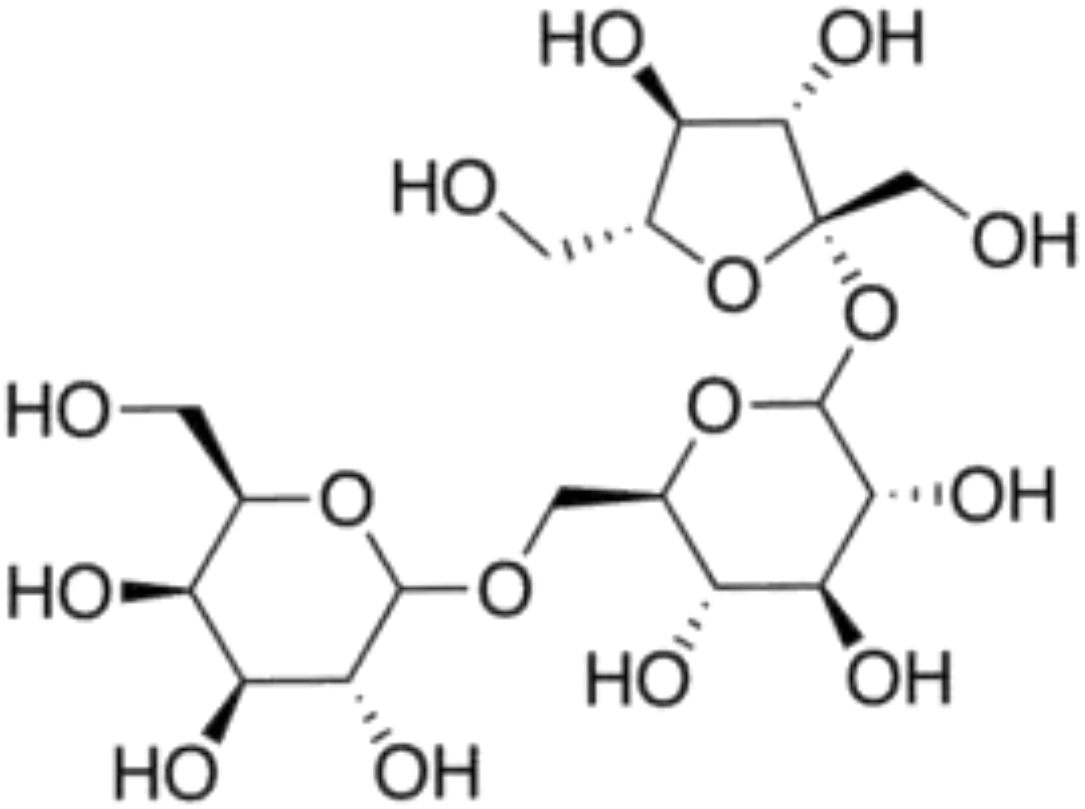

Figure .2.1: The structural formula of raffinose. Source: (Anna Macleod, 1957)

2.4.2 Sources of Raffinose

2.4.2.1 Plant sources of Raffinose

Raffinose is a trisaccharide composed of galactose, fructose, and glucose. It can be found in beans, cabbage, brussels sprouts, broccoli, asparagus, soybeans, legumes, other vegetables, and whole grains. Raffinose can be hydrolyzed to D-galactose and sucrose by the enzyme α-galactosidase (α-GAL), an enzyme not found in the human digestive tract. (http.www.righthealth.com/corp/doingright; Townsend and Pitchford, 2012).

2.4.2.2 Other sources of Raffinose

Other sources are honey, brown sugar, sugar beet and molasses.

Honey: It is a sticky golden brown fluid produced by bees from the nectar of flowers. Raffinose occur naturally as the main sugar in this sweetener used for cooking, it could also be added to tea, coffee or cereal or it can be spread on bread. The toxins found in pollen include lactose, raffinose, stachyose, xylose, arabinose, galactose, galacturonic acid, glucuronic acid, and pectin. However, the extraction techniques of the chemist may yield different compounds than are extracted by bees during the digestion of pollen (Barker, 1997; Marero *et al.*, 1998; Townsend and Pitchford, 2012).

Stored pollen becomes less nutritious. This decline is usually attributed to loss of dietary essentials. The possible slow release of bound toxic sugars or oxidation of pollen sugars to more toxic uronic acids needs consideration (Barker, 1997; Kokiladevi *et al.*, 2005). Pollen undoubtedly supplies dietary essentials, but the sugars in some samples may exceed tolerable levels. These toxins need to be diluted or degraded to a safe level before bees can live on the pollen. When bees pack pollen with nontoxic sugar, such dilution can be accomplished (Barker, 1997; Kokiladevi *et al.*, 2005).

Besides providing insurance against starvation during a nectar shortage, production and storage of honey by bees seems to be a mechanism to dilute toxins. Health-promoting compounds found in honey could make this ingredient a more attractive option for food makers currently using bulk sweeteners such as high-fructose corn syrup and looking to jump on board the growing health foods trend. Other sugars like glucose, fructose and raffinose also enhance calcium absorption; it also contains a number of antioxidant components that act as preservatives which may as well serve as replacement for some synthetic antioxidants. Researchers say that honey may be a

healthier alternative to corn syrup due to its higher level of antioxidants, compounds which are believed to fight cancer, heart disease and other diseases (Barker, 1997; Marero *et al.*, 1998; Kokiladevi *et al.*, 2005).

Brown sugar: It is a soft liquid or dark brown sweetener made from refined white sugar combined with mild refined molasses, used in cooking or making beverages. Raffinose is the only commonly-occurring sugar found in brown sugar when recovered from the sugar industry which is usually discovered during different conditions of chemical analysis. The sugar raffinose occur as elevated bound galactose, characteristic of this sugar which has a composition somewhat differing from the average honey constituent or arises from traces of sugar molasses, though the latter appears more likely closer (Sanni and Knights, 1984; Marero *et al.*, 1998 ; Townsend and Pitchford, 2012).

The bound galactose (in raffinose) is a constituent of all brown sugar which represents small and variable amounts of sugar recovered from the industry. This is usually detected by the optical polarization of the samples of the bound galactose (Sanni and Knights, 1984).

Sugar beet: Beet has different varieties with whitish conical root that is an important commercial source of sugar. It is a plant with a large swollen root that is used as vegetable, animal feed and also for sugar production. The roots of the beet are cut into chips and crushed to remove the juice; it undergoes chemical processes and purification in the industry and is then made into sugar. Raffinose is usually the major galactosyl-sucrose oligosaccharide, a significant component in sugar beet (Beutler, 1988). Like sucrose, raffinose is precitated by lime in the saccharate process and is retained in the syrups because of continued recycling of the juices in the sugar factory (Beutler, 1988; Marero *et al.*, 1998; Kokiladevi *et al.*, 2005; Townsend and Pitchford, 2012).

The elimination of this unwanted substance is in the hands of the enzymatic action of other carbohydrate results in the industry. Raffinose content of beet is affected by cold weather, freezing, storage, environment during growth and genetic composition of the beet (Beutler, 1988; Marero *et al.*, 1998; Kokiladevi *et al.*, 2005). Raffinose is one of the many unwanted melassigenic substances, commonly called impurities, which interferes in the recovery and refining of sucrose. It is a dextrorotatory, trisaccharide compound with very little commercial value (Beutler, 1988).

Sugar Molasses: It is the thick sticky sweet syrup produced during the refining of raw sugar which ranges in colour from dark brown to gold. It is a valuable by-product of the sugar industry being used in the manufacture of etyl alcohol and food flavouring. It

can be made into granules, powdered or lump sugar. Raffinose is also present in sugar molasses. It is usually the major galactosyl-sucrose oligosaccharide, a significant component in sugar molasses (Barker, 1997; Kokiladevi *et al.,* 2005).

Raffinose is one of the many unwanted melassigenic substances, commonly called impurities, which interferes in the recovery and refining of sucrose. It is a dextrorotatory, trisaccharide compound with the chemical makeup and has very little commercial value. Like sucrose, it is precipitated by lime in the saccharate process and is mostly retained in the syrups because of continued recycling of the juices in the sugar factory (Beutler, 1988; Kokiladevi *et al.,* 2005; Townsend and Pitchford, 2012).

2.4.3 Raffinose Metabolism into Simple Sugars

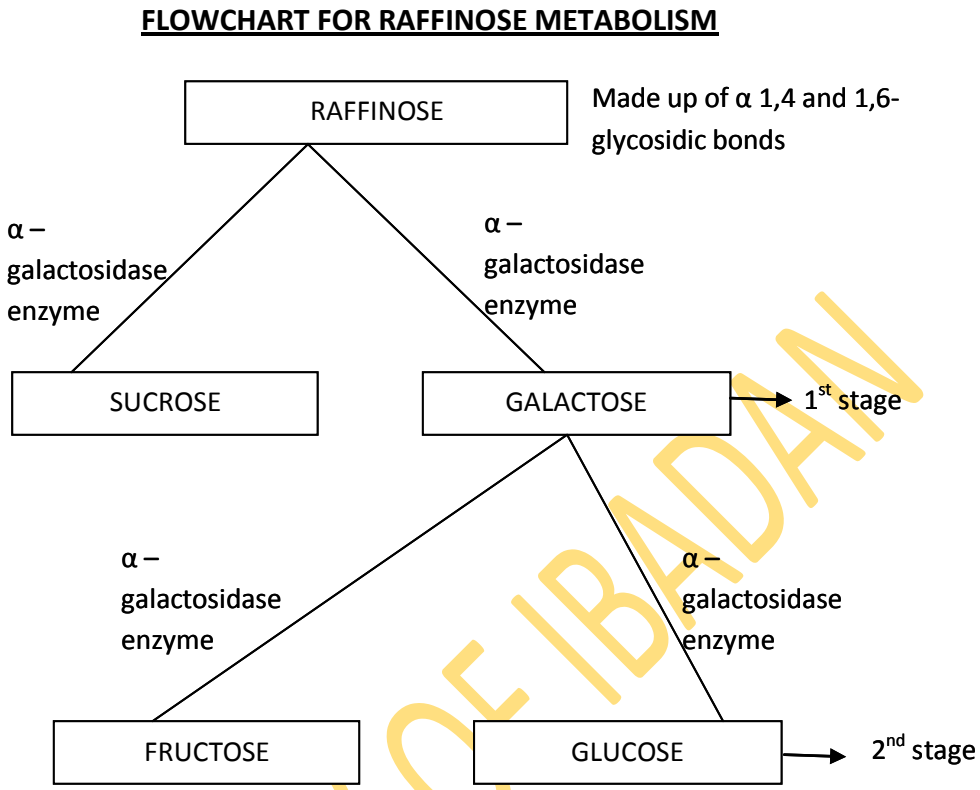

Figure 2.2: Flowchart for Raffinose Metabolism

PRINCIPLE:

Raffinose is hydrolysed to D-galactose and sucrose by α-galactosidase (α-GAL). α-GAL also hydrolyses other α-galactosides such as stachyose, verbascose and galactinol [1-O-(α-D-galactosyl)-*myo*-inositol], if present. The enzyme does not cleave β-linked galactose, as in lactose.

(α-galactosidase)

(1) Raffinose + H_2O ----------→ D-galactose + sucrose

Interconversion of the α- and β-anomeric forms of D-galactose is catalysed by galactose mutarotase (GalM).

The measurement of raffinose as D-galactose liberated is an alternative to the measurement as D-glucose liberated through the action of α-galactosidase plus β-fructosidase (Barker, 1997).

2.4.5.1 Weaning Substituents by Human

(a) Weaning Foods

Scientifically, it has been proved that breast milk is the perfect food for the infant during the first six months of life. It contains all the nutrients and immunological factors an infant requires to maintain optimal health and growth (UNICEF, 1999; MacDonald *et al.*, 2012). Furthermore, breast milk also protects infants against the two leading causes of infant mortality, upper respiratory tract infections and diarrhoea (UNICEF, 1999; MacDonald *et al.*, 2012). However, at the age of six months and above when the child's birth weight is expected to have doubled, breast milk is no longer sufficient to meet the nutritional needs of the growing infant. Nutritious complementary foods also known as weaning foods. This category of food typically covers the period from six to twenty four months of age in most developing countries are therefore introduced (Mariam, 2002; WHO/OMS, 2000).

Weaning food is described as any food - liquid, semi-solid or solid - that is introduced to infants from four months other than breast milk (Ameny *et al.*, 1994). Exclusive breast feeding is usually adequate up to three to four months of age, but after this period, it may become increasingly inadequate to support the nutritional demands of

the growing infant. Thus, in a weaning process, there is always the need to introduce soft, easily swallowed foods to supplement the infants feeding early in life (Onofiok and Nnanyelugo, 1998; MacDonald *et al.* 2012). On the other hand, nowadays, due to the reduced consumption of breast milk, important nutrients such as proteins, zinc, iron and B-vitamins are likely to be deficient in the contemporary diet of the affected infants (La Leche League International, 2002). If this development is not well-handled during this crucial growth period, it can lead to malnutrition. Poor feeding practices and/or shortfall in food intake have been identified as the most important direct factors responsible for malnutrition and illness amongst children in Nigeria (Mariam 2002; NNN, 2000).

The weaning process therefore, may be gradual, lasting for some time until the infant is introduced to the family diet. Abrupt weaning, on the other hand, i.e. the introduction of the infant straight to the family menu may create a problem that the mother may not be able to handle. The child may not be able to eat enough of the adult diet that will meet his /her nutritional needs. At times, the digestive system of the child may not have been well developed to break down the composition of such adult diet (Onofiok and Nnanyelugo, 1998; Mariam, 2002: Gray *et al.*, 2012).

In West Africa, the first solid food and the most popular weaning food is a thin cereal gruel that is called by different names depending on the type of cereal or the country. In Nigeria for example, the usual first weaning food is called *pap, akamu, ogi or koko* and is made from maize (*Zea mays*), millet (*Pennisetum americanum*), or guinea corn (*Sorghum spp.*); (King *et al.*, 1987).The low-income earners use these foods principally because they cannot afford the factory processed baby foods. But the importance and advantages of this gruel to young infants is more than this and clearly outweighs that of the imported or canned foods.

(b) Problems associated with preparing adequate weaning food for infants

Problems usually arise from the way the food is being introduced to the child. While some mothers believe that additives such as milk should be added as supplement, others, especially from the low-income group, do not see a need for this at all. They do not make special effort to adequately prepare the child's weaning diet. And so, such are usually monotonous, boring, unhygienically prepared and lacking sufficient nutrient for the growth of the child (Onofiok and Nnanyelugo, 1998; Mariam, 2002).

Another problem that is associated with weaning foods in West Africa is the problem of diarrhoea and diarrhoea-related diseases that are highest at this level, especially in children that are less than one year. At this stage, these foods are gradually

introduced to supplement and succeed breast feeding. This also plays a major role in child's mortality and morbidity. This is because due to unhygienic and inappropriate preparation of weaning foods, episodes of diarrhoeal diseases are highest with the combination of nutrient malabsorption and malnutrition (Mariam, 2002: Gray *et al.*, 2012).

In addition to bacterial intestinal pathogens causing infectious diarrhoea, viral and parasitic infections may also bring about chronic or acute diarrhoea or may contribute to malnutrition. The food, food handlers, water and utensils used in preparing and serving the food to infants play an important role as vectors for infective diarrhea (Itotia *et al.*, 1987; Mariam, 2002). Although any human being risks a food-borne infection after consumption of contaminated food or water, epidemiological evidence shows that the groups that are particularly at risks are those who are weak or in a poor nutritional condition. Such persons are mainly infants, young children, the sick and elderly (Chen, 1983 *et al.*,; Mariam, 2002).

Most weaning foods are prepared by boiling its ingredients in water. Although most microbial contaminants will be inactivated as a result; heat-resistant parasitic cysts and bacterial endospores may survive. Even more important, recontamination of the cooked weaning food is very likely to occur from utensils, handling, insects, feaces, etc before it is consumed. This may however lead to microbial growth or turn wholesome food into a health hazard one depending on the nature of the contamination, the culture medium and its storage conditions (Food and Nutrition Bulletin, 1989; MacDonald *et al.* 2012).

Rowland *et al.* (1987) stressed that weaning foods for tropical use should be reasonably resistant to bacterial growth for at least the first one to two hours of feeding. Possible interventions for diarrhoeal disease control include promoting improved practices for the preparation of weaning foods that are nutritionally adequate, ready-to-feed and easy to be preserved safely without getting contaminated (Nout *et al.*, 1989). Lactic acid fermentation of cereal products is, however, a possible solution in this regard. This is, however, widely practiced in African countries as a household-level food technology. Products such as *pap/ ogi/ eko/ akamu*, *mahewu* and *uji* are popular sour gruel and are widely used throughout the day by young and old. The acceptance of this type of products offers opportunity to stimulate the consumption of fermented products for hygienic reasons and because LAB have been implicated in being safe and their use

as probiotics has been described as a possible solution to contamination of weaning foods(Nout *et al.,* 1989; MacDonald *et al.* 2012).

Furthermore, one of the problems encountered in the bid to get adequate diet for children of weaning age is that of the dietary bulk properties of cereals that are used as weaning food. The popular staple food is either too thick for the children to feed on or the nutritional/energy need of such infants are not met. Children usually prefer the weaning food as thin gruel, but in the bid to increase the energy and nutrient density, which is appreciated by adults, the food becomes stiff and difficult for the children to consume (Mosha and Svanberg, 1990). The dual problem of high volume characterized by low energy and nutrient density and stiffness is however, a problem that makes it difficult for children of weaning age to consume and meet their energy and nutrient requirement (Lorri, 1993).

According to MacDonald *et al.* (2012), careful weaning planning is particularly important, there are should be evidence-based weaning guidelines and studies assessing the introduction of semi-solid foods before the introduction of solid food for healthy infants. Also, biological, maternal, infant, social and environmental factors are also required. This will help provide evidence for the effect of protein substitute on appetite and help in the development of evidence-based guidelines.

Different methods have been suggested to solve this problem. Application of processing techniques which partially or completely breakdown the starch granules is one method. This reduces the bulkiness during cooking (Walker, 1990). Other methods include extrusion cooking, use of germinated grains; these methods improve the digestibility and palatability of the gruel (Jansen *et al.*, 1981). Malting is also another method of reducing the viscosity of the starch-base cereal gruel and it has been found to permit better digestibility, increase vitamin levels and increase the amino acids and relative nutritional value of the food (Desikachar 1983; Mosha and Svanberg, 1983).

Several authors have reported that different fermentation techniques which combine the use of ‘power flour’ and a small amount of lactic starter culture provides a similar improvement in the dietary bulk properties (Adeyemi and Beckley, 1986;Lorri and Svanberg,1993).

Cereal and legumes which are also used as weaning foods also contain significant amount of antinutrients, dissacharides and oligosaccharides. These further lower their nutritional quality. Legumes contain some natural toxicants which include tannins, phytic acid, protease and trypsin inhibitors, saponins, metal chelates, cyanogens, isoflavonoids,

phytoalexins, flatus factors etc (Pariza, 1996). Most cereals like sorghum and millet also contain some appreciable amount of phytate, tannins, protease and trypsin inhibitors and polyphenols. Some of these substances reduce the nutritional value of the food by interfering with mineral bioavailability and digestibility of proteins and carbohydrates (Salunkhe *et al.*, 1990; Haard, 1999; MacDonald *et al.* 2012). Since legumes are usually consumed along with cereals in form of additives, or as protein source, proper processing of these food substances should therefore be encouraged to eliminate these anti-nutrients before they are consumed (Reddy and Pierson, 1994).

Another major problem with the use of legumes as weaning food is the presence of complex oligosaccharides and dissacharides such as lactose,galactose, raffinose, stachyose, verbascose and galactinol etc.These are complex sugars that are not easily broken down in a child's digestive tract and so it results in indigestibility, intolerance and irritation of different sorts. This subsequently leads to gas formation, discomfort, flatulence and abdominal pain in children. If these persist on a continuous basis, it leads to diarrhoea, malnutrition, weight loss, nutrient deficiency and a sickly child (Buheloees, 2007).

Even though the problems highlighted above abound in the preparation of adequate weaning food for children, LAB is able to solve these problems by their metabolic processes in the fermented foods that contain them. This is why these fermented cereals are being encouraged in different part of West Africa and mothers are being encouraged to take time to prepare the food themselves (FAO, 2004). Raffinose is a non-reducing hetero-oligosaccharide that can be metabolized by certain groups of LAB (Hassan, 1998). The LAB obtains their energy by the ability to ferment oligosaccharide such as raffinose and produce metabolites in the medium of growth. They produce the enzymes -alpha and beta galactosidases - that are able to breakdown raffinose into small reducing sugar units that can be absorbed by the body. Such nutrients are identified as encodes the enzymes necessary for transport of the substrates for their subsequent conversion to an intermediate in glycolysis (Odunfa, 1985, Steinkraus *et al.*, 1995).

Ability to ferment or metabolise these complex sugars by some LAB however, is a plasmid-linked trait, and so when the plasmids of such organisms are removed by molecular means, they lose the ability to breakdown this complex sugars or the rate of conversion to simple monosaccharide unit is reduced. Furthermore, the reducing sugars that are produced when the plasmid is lost are usually small.

2.4.5.2(a) Food Supplement and Fortification

Food fortification is the public health policy of adding micronutrients (essential trace elements and vitamins) to foodstuff to ensure that minimum dietary requirements are met (Honein *et al*., 2001). Simple diets based on staple foods with little variation are often deficient in certain nutrients, either because they are not present in sufficient amounts in the soil of a region, or because of the inherent inadequacy of the diet. Addition of micronutrients to staples and condiments can prevent large-scale deficiency disease in these cases (Honein *et al*., 2001; Gray *et al.*, 2012).

Several ranges of food supplements have been recognized, including additives which repair a deficit to "normal" levels, additives which appear to enhance a food and supplements taken in addition to the normal diet.

Many food scientists today disagree with the notion that foodstuffs need supplementation, but accept that - for example - added calcium may provide benefit for women or growing children (Crane *et al*., 1995; MacDonald *et al.* 2012). On a more controversial level, but well founded in scientific basis, is the science of using foods and food supplements to achieve a defined health goal. A common example of this use of food supplements is the extent to which body builders will use amino acid mixtures, vitamins and phytochemicals to enhance natural hormone production, increase muscle development and reduce fat storage (Badamosi *et al.***,** 1995).

Need for fortification of weaning foods cannot be overemphasized. This is because traditional weaning foods in Nigeria are formulated based on local staple usually cereal grains such as maize, sorghum, millet and rice and roots and tubers such as yam and cassava .To be suitable for the feeding of young children, the cereals are prepared in liquid form by diluting with a large quantity of water, thereby resulting in a large volume with low energy and nutrient density (Mbata *et al.,* 2006). Despite the reported improvement in the nutrient status of germinated and fermented cereal based diets in sub-Saharan Africa, the nutrient needs of infants and sick adults are still not being met. Studies have therefore shown the need for supplementation and fortification of traditional fermented maize porridge with legume (Mbata *et al.,* 2006).

Moving on from this reasonably accepted usage, there is increasing evidence for the use of food supplements in established medical conditions. This nutritional supplement using foods as medicine (nutraceuticals) has been effectively used in treating disorders affecting the immune system up to and including cancers. This goes beyond the

definition of "food supplement", but should be included for the sake of completeness (Badamosi *et al.*, 1995; http://en.wikipedia.org/wiki/Dietary_supplement).

2.4.5.2(b) Food supplements

There are several main groups of food supplements, which can be considered according to Badamosi *et al.* (1995). They are Proteins, Vitamins and co-vitamins, Essential minerals, Essential fatty acids, Essential amino acids, Glyconutrients and Phytonutrients (http://en.wikipedia.org/wiki/Dietary_supplement).

2.4.5.2 (c) Examples of fortified foods

Soybeans (*Glycine max*) is usually added to cereals that are made for weaning children, this has been used over the years in Nigeria, especially in the South West Nigeria, where '*Ogi*' is fortified with different forms of modified soybeans (Badamosi *et al.*,1995). Roasted cowpea has been used as the major ingredient for the fortification of traditional fermented maize porridge used for a pilot study intervention in twelve communities in Kwara State, Nigeria (Guptill *et al.*, 1993; Mariam, 2002). Also, Nti and Plahar *et al.* (1995) reported the chemical and biological characteristics of a maize-based traditional weaning food in West Africa that is supplemented with 30% precooked-cowpea.

Ogi is a fermented sour corn pap or porridge widely used as a breakfast food and weaning food among several *Yorubas* in the western states of Nigeria. Akinrele (1971) found that the biological quality of the protein in *ogi* was so poor that it did not support growth in rats, but when it was fortified with 30% heat-treated whole soy flour, the protein efficiency ratio (PER) increased threefold, making the protein usability almost equal to that of casein.

Beans serve as the largest single contributor to total protein intake in many rural and urban families in West Africa (Onigbinde and Akinyele, 1983). The application of cowpea-fortification to fermented cereals porridges has been reported to increase the nutritive value and total nutrient composition of such foods (Afoakwa, 1996; Sefa-Dedeh *et al.*, 2000; Mariam; 2002).

Another low-cost, protein-rich and energy-rich weaning food of acceptable quality was prepared using maize-cowpea and crayfish as the major ingredients (Abbey *et al.*, 1998). Folic acid is also added to flour in many industrialized countries, and has prevented a significant number of neural tube defects in infants (Fernandez *et al.*, 2002).

It is, however, not uniform in its application, with more intake of folic acid through fortified flour among those who were already receiving high amounts through their diet (Oguntona and Akinyele, 2000).

Soy-based infant formula (SBIF) is used for infants who are allergic to pasteurized cow milk proteins. It is sold in powdered, ready-to-feed, and concentrated liquid forms. Some reviews have expressed the opinion that more research is needed to determine what effect the phytoestrogens in soybeans may have on infants. Diverse studies have concluded there are no adverse effects on human growth, development, or reproduction as a result of the consumption of soy-based infant formula (Hymowitz, 2012).

In Ghana, one of the traditional weaning foods is a roasted-maize-based porridge popularly called *Tom Brown*. Like many other cereal-based weaning foods used in the region, *Tom Brown* is relatively low in protein. To enhance its nutrient content, the Ministry of Health and the United Nations Children's Fund introduced a product called *weanimix*, which is a fortified *Tom Brown* composed of 75%-80% maize, 10%-15% cowpeas or soybeans, and 10% groundnuts (Nagai *et al.*, 2012).

Niacin has been added to bread in the USA since 1938 (when voluntary addition started), a programme which substantially reduced the incidence of pellagra (Oguntona and Akinyele, 2000) etc.

2.5 (a) Use of Soybeans

Soybeans (*Glycine max L.*) as a legume, is widely grown in the Middle Belt Region of Nigeria (Oyenuga, 1978). It is rich in high level dietary protein (40%, w/w), its utilization by the local population compared to cowpea is still limited (Castillo *et al.*, 1990). It contains a high level of antinutritional factors such as tannin, trypsin inhibitor and phytic acid. There is also the presence of some oligosaccharide such as raffinose, stachyose and verbascose which possess flatulence – inducing properties (Castillo *et al.*, 1990).

Many food items have been produced from it such as flour, milk, fermented products, cake which are used as supplement or additives but the problem of flatulence, gas production and floating still persist (Gray *et al.*, 2012). Efforts were later directed by various workers at reducing the flatulent properties by the hydrolysis of the oligosaccharides by treatment of the product with the enzyme ά – galactosidase (Gray *et*

al., 2012). Alpha-galactosidase is known to breakdown raffinose at the ά – 1,4 glycosidic bond whole ß–galactosidase break it down at ά-1-6 position (White *et al.*, 1985).

2.5(b) Soybeans as Good Protein Source

As in most other developing countries, the high cost of fortified nutritious, proprietary complementary foods is always, if not prohibitive, beyond the reach of most Nigerian families. Such families often depend on inadequately processed traditional foods consisting mainly of un-supplemented cereal porridges made from maize, beans, soybeans, sorghum and millet. In view of this, appropriate processing and blending of locally available food commodities have been carried out and researched into, by a number of workers (Badamosi *et al.*, 1995). Such blends have been found to improve nutrient density of the complementary food as well as nutrient intake, which results in the prevention of malnutrition problems.

This approach would require knowledge about the nutritive values of a variety of local food commodities, indigenous to the affected communities. A number of cereals and legumes that are readily available in Nigeria have been found to have nutrient potentials that could complement one another if properly processed and blended (Oguntona and Akinyele, 2000; Mariam, 2005). Therefore, it is imperative that efforts to formulate composite blends and scientific studies are carried out to ascertain the nutritive adequacy of these locally available blends (cereal and legumes) for possible use as complementary foods, especially by the rural and poor urban mothers during weaning period (Oguntona and Akinyele, 2000).

Cereals are the main ingredients in most of the traditional weaning foods. However, since the nutrient density of these weaning foods is low, it is almost impossible for small children to meet their needs for calories and protein from the amount that they can ingest. Sources of good quality protein such as meat are expensive for low-income families. Many studies have examined how to improve the nutritional quality of traditional weaning foods using affordable staples such as legumes (Nagai *et al.*, 2012).

2.6 Anti-Nutritional Factors (ANFs)

An **anti-nutritional factor** is a substance which, when present in human or animal foods, reduces growth. Examples are phytate, tannins, protease inhibitor (notably soybeans trypsin inhibitor) and excessive dietary fiber. Food can naturally contain antinutrients that, in large quantities, can be harmful to the body when consumed. It is

therefore important to know about potential antinutrients (United Nations World Food Programme, 2009).

Antinutrients play two important roles in the plants in which they are present. First, their negative effect on the nutritional quality of the varieties of the food in which they are present, and the attendant toxic effects. The second is the role they play in certain defence mechanisms in such plants against pathogens and pests. Hence, the need for their removal or reduction to certain safe levels factors before being consumed by man or animals (Martin *et al.*, 1991; Hymowitz, 2012).

2.6.2 Types of ANFs

- **Protease inhibitors** which inhibit the activity of trypsin, chemotrypsin and other proteases. They are found in legumes such as beans and peas, but also in cereals, potatoes, and other products. Their presence results in impaired growth and poor food utilization (Salunkhe *et al.*, 1990). They also cause growth inhibition by interfering with digestion, causing pancreatic hypertrophy and metabolic disturbance of sulphur and amino acid utilization (Reddy and Pierson, 1994).
- **Tannins** are found in soybeans, peanuts, sugar beets and others. They are oligomers of flavan-3-ols and flavan-3, 4-diols which occur widely in legumes (Haard and Chism, 1996). These compounds are concentrated in the bran fraction of legumes. Tannin-protein complexes can cause inactivation of digestive enzymes and reduce protein digestibility by interaction of protein substrate with ionisable iron (Salunkhe *et al.*, 1990). The presence of tannins in food can therefore, lower feed efficiency, depress growth, decrease iron absorption, damage the mucosal lining of the gastrointestinal tract, alter excretion of cations, proteins and essential amino acids (Reddy and Pierson, 1994).Toxic effects have also been shown to cause the impairment of nitrogen digestibility by the negative interaction of tannins with the foods that are being consumed (Ling, 2007).
- **Phytic acid/phytate** occurs in several vegetable products. Its presence may affect bioavailability of minerals, solubility, functionality and digestibility of proteins and carbohydrates. (Salunkhe *et al.*, 1990). Phytate chelates divalent cations such as calcium, magnesium, zinc and iron thereby reducing their availability to humans when such foods that contain them are consumed (Haard, 1999).

2.6.3 Sources of Anti Nutritional Factors

Anti-nutritional factors are classified as those naturally present in the grains or cereals and those that are due to contamination which may be of fungus origin or may be related to or/and other environmental influences. Others also get to the grains as a result of .pesticides/insecticides used for preservation on the field/farm or during storage. These factors modify the nutritional value of the individual grains and several of them have very serious consequences (FAO, 1995).

Defence Mechanism: Other antinutritional factors occur naturally in plants and are used as defence mechanisms against animal/mechanical attacks e.g. some varieties of sorghum contain high tannin in the grain and this is found to be bird resistant(Barns, 1971; Tipton *et al.,* 1970). Tannis are the most abundant phonetic compound in brown bird resistant sorghum. During maturation the brown sorghum grain develop astringence properties which become resistant against bird and grain mould attack (Tipton *et al.,* 1970). They are either present in the seed coat, bran, endosperm, bristles, pericarp or other part of the seed.

Also, the bran and aleuronic layers in sorghum grain serve as a major reservoir of phytate and total phosphorus (Sankara Rao and Deosthale, 1983).

Water Sources: Some other chemicals such as fluoride and other trace elements that confer health hazard to the grain may also get to it through different water sources while growing in the farm (Lakshmanzh and Srikatia, 1977).

Soil Uptake: Trace elements like copper, fluoride and silicon molybdenum may be hitherto present in the soil before planting and there may be uptake or absorption by the plant (Underwood, 1971).

The presence of these trace elements in significant amount in the soil accumulates in different parts of the plant and its consumption posses a high health hazard (Underwood, 1971).

Mycotoxins: Another source of ANF is mycotoxin. Cereals like sorghum and millets are susceptible to fungal growth and mycotoxins production under certain environmental conditions. Mycotoxins not only threaten consumer health but also affect food quality and quantities, causing huge economic losses (FAO, 1990). Storage fungi, mostly of the genera *Aspergillus,Fusarium* and *Penicillium* are found on food grain stored with moisture content greater than 13% (Sauer, 1988). Mouldy sorghum hair head were shown to be contaminated with aflatoxins B and G (Alpert *et al.,* 1971).

Infestation i.e. insect damage during storage is also another way of introducing ANF to grains/cereals. This does not only result in food loss but also affects the nutritional quality of such foods (Kapu *et al.,* 1989). Alpert and others *(*1971) have observed reduction in protein and starch digestibility on grain infestation in wheat, sorghum and maize.

Insecticides/Pesticides: These are used to prevent animal or insect infestation in the field or during storage. The content/amount confers a health hazard to consumers of such food substances if it is not used within the appropriate recommendation. Some pesticides are been removed from market as a result of this (Gamallin 20) while farmers need to be tutored on its uses (Alpert *et al.,* 1971).

Fertilizers/Crop enhancers: Some may contain chemicals that affect the crops adversely. This must be used following the Manufacturers' details about quantities to be used and the time interval between the period of use and when such foods can be consumed.

There are different methods however that are available which may remove/reduce the ANF that may be present naturally or as a result of contamination.

2.6.4 Implication of antinutritional component of Soybeans

There are a number of components present in soybeans that exert a negative impact on the nutritional quality of the protein. Among those factors that are destroyed by heat treatment are the protease inhibitors, tannins, phytate and lectins (Roem, 2000).

Protease inhibitors exert their antinutritional effect by causing pancreatic hypertrophy/hyperplasia, which ultimately results in an inhibition of growth.

The lectin, by virtue of its ability to bind to glycoprotein receptors on the epithelial cells lining the intestinal mucosa, inhibits growth by interfering with the absorption of nutrients (Ika *et al.,* 1999). Of significance also are the antinutritional effects produced by relatively heat-stable factors, such as goitrogens, tannins, phytoestrogens, flatus-producing oligosaccharides, phytate, and saponins (Roem, 2000).

Other diverse but ill-defined factors appear to increase the requirements for vitamins A, B12, D, and E. The processing of soybeans under severe alkaline conditions leads to the formation of lysinoalanine, which has been shown to damage the kidneys of rats (Ika *et al.*, 1999). This is not generally true, however, for edible soy protein that has been produced under milder alkaline conditions. Also meriting consideration is the

allergenic response that may sometimes occur in humans, as well as calves and piglets, on dietary exposure to soybeans (Gilani *et al*., 1998).

There is thus the need for natural removal method such as cooking, roasting, dehulling and most importantly, fermentation by Lactic Acid Bacteria. The growth-inhibiting effect of feeding pretreated and modified soybeans to humans and young animals was reduced by the factors mentioned above and they become less detrimental to health. It has also been shown that soybean treatments improved growth performance and improves the nutritional quality of the foods in which they are added (Shalini, 2006; Buheloees, 2007).

2.7 Consequences of Soybeans fortification of Weaning Foods

2.7.1 The Problem of Bloating, Gas Production and Flatulence

Raffinose intolerance is the inability or insufficient ability to digest raffinose, a complex sugar found in beans and some leguminous products. Raffinose intolerance is caused by a deficiency of the enzymes alpha and beta galactosidase which are not produced by man naturally but by certain groups of enteric microorganisms and LAB which is able to break down this sugar into simple form; first into galactose, this is then broken down into fructose and glucose which are then absorbed into the bloodstream (Heyman, 2006; Hymowitz, 2012).

Not all people with this deficiency have digestive symptoms, but those who do may have raffinose intolerance. This is common in young children that use foods that contain the complex sugars as weaning foods and in some adult whose immunity have been suppressed or compromised. Most adult with raffinose intolerance can tolerate some amount of raffinose in their diet (Mbata *et al*., 2012).

2.7.2 What are the symptoms of raffinose intolerance?

People with raffinose intolerance may feel uncomfortable thirty minutes to two hours after consuming beans and leguminous products. Symptoms range from mild to severe based on the amount of raffinose consumed and the amount a person can tolerate. Common symptoms include abdominal pain, abdominal bloating, gas production, diarrhoea, nausea and vomiting (Heyman, 2006; Buheloees, 2007).

The problem defined

The trisaccaharide raffinose cannot be absorbed through the wall of the small intestine into the blood stream, so in the absence of the enzyme that can digest this, raffinose present in the foods that are consumed remains uncleaved and passes into the colon (Heyman, 2006; Hymowitz (2012). The operons of the enteric bacteria quickly switch over to raffinose metabolism and the resulting *in-vivo* fermentation produces copious amount of gas which is a mixture of hydrogen, carbon-dioxide and methane (Heyman, 2006; Hymowitz (2012). This in turn may cause a range of abdominal discomfort, symptoms including cramps, bloating and flatulence in children. In addition, as with other unabsorbed sugars, such as raffinose, lactose, mannitol, sorbitol, its presence and its fermentation products raises the osmotic pressure of the colon thereby causing discomfort (Buheloees, 2007).

According to Hymowitz (2012) the principal soluble carbohydrates of mature soybeans are the disaccharide sucrose , the trisaccharide raffinose, the tetrasaccharide stachyose and pentasaccharide verbascose one sucrose connected to two molecules of galactose. While the oligosaccharides raffinose and stachyose protect the viability of the soybean seed from desiccation, they are not digestible sugars, and therefore contribute to flatulence and abdominal discomfort in humans and other monogastric animals. Undigested oligosaccharides are broken down in the intestine by native microbes, producing gases such as carbon dioxide, hydrogen, and methane. As a result of this indigestibility, diarrhoea results. If such foods are taken on a regular basis by children, especially as weaning foods and as such cannot be metabolised, weight loss, nutritional deficiency and malnutrition results in growing children (Mariam, 2002). Sugars in soybean seed affect soyfood quality, digestibility, and nutritional values.

Soyfood such as soymilk, *tofu*, and *natto* is considered as healthy diets, and consumption is highly recommended by nutritionists and medical doctors. In *tofu*, soymilk, *natto*, and many other soyfood products, desirable sugars including glucose, fructose, and sucrose contribute to the favourable sweet taste and are ready-to-digest, while raffinose and stachyose are indigestible and cause undesirable flatulence and diarrhoea (Hymowitz, 2012).

It is estimated that some children in Asia or Africa may suffer from either primary, secondary or environmentally- induced lactose or raffinose intolerance because of poor - lactose rich food in the diet or an infection by a parasite called ‘giardia’ (Buheloees, 2007). Furthermore, about 75% of adult worldwide show some decrease in

lactase and galactosidase activity during adulthood. The frequency of decreased lactase activity ranges from as little as 5% in Northern Europe, up to 71% for Southern Europe, to more than 90% in some African and Asian countries.

2.7.3 Raffinose and Lactose Intolerance, Indigestibility and Malabsorption

Raffinose is a trisaccharide composed of galactose, fructose and glucose. Raffinose is an endogenous metabolite but may be obtained from the consumption of partially fermented molasses, brown sugar and honey (Sharma *et al.*, 1995; Gray *et al.*, 2012). LAB is able to utilize these sugars as carbon source for growth. It is also found in many vegetables and legumes like beans, cabbage, soybeans, brussels, sprouts, broccoli, asparagus, other vegetables, and whole grains.

In the lower intestine, raffinose is fermented by gas-producing bacteria which possess some enzymes that catalyses the breakdown of raffinose (Thompson, 1998). Raffinose can be metabolized by enteric organisms and LAB. Foods containing raffinose are sometimes taken as food additives, fortification or supplement which are taken either to add to the nutrient value of the food. They may be taken because of their health benefits, as whole food or supplement to normal diet (Pariza, 1996; Honein, 2001; http://en.wikipedia.org/wiki/Dietary_supplement).

Lactose on the other hand is a disaccharide that is hydrolyzed into galactose and lactose by the enzyme lactate which is also produced by LAB. Various works have been shown that described that some children and some adult suffer from inability to metabolise raffinose and from lactose intolerance (Honein, 2001). This results in indigestibility and malabsorption. Raffinose intolerance is the inability of some people to metabolize raffinose. This is not because of the length of the chain but because of the nature of the bonds in the polymerizing units of raffinose (Pariza, 1996).

2.8 Importance of Fermentation in Reduction of Oligosaccharides and Antinutritional Factors in Foods

Fermented foods are estimated to constitute about a quarter of the foods consumed worldwide. In Africa, a majority of the fermented foods are produced at household level and hygiene is a major concern. A wide variety of foods are fermented, including milk, root crops, meat and fish, but the foods of greatest relevance fed to young children are produced by fermentation of cereals, grains, milks and pulses (Badamosi *et al.*,1995; Nnakwe,1995; Modu *et al.*,2004).

In Nigeria, for example, *Ogi* is used as weaning food in children. They have been confirmed to be safe against food- borne diseases because of their low pH. LAB starter culture used in fermentation produce antimicrobial compound such as bacteriocin, hydrogen peroxide, formic acid, acetic acid, lactic acid and diacetyl (Motarjemi and Nout, 1996; Oguntona and Akinyele, 2000; Modu *et al.*, 2004).

In many communities in Africa, the beginning of weaning young children off breast milk is associated with an increase in diarrhoeal episodes. This is because standards of hygiene are low in some of these communities. Post-cooking contamination and the use of contaminated water during preparations are often cited as causes of diarrhoea during weaning (Livingstone *et al*., 1993; Latham *et al*., 1997; Onofiok and Nnayelugo, 1998; Mariam, 2004).

Several authors have highlighted the importance of adequate nutritional quality and hygiene during the preparation of weaning food and also the link between infection and nutrition (Svanberg, 2004). Lactic fermentation is thus widely believed to be protective against food borne disease and is usually recommended as a cheaper way of preparing weaning food (Wondimu and Malleshi, 1996; www.file://F:agbaje2.htm).

Fermentation technology has been employed over the years to reduce the antinutritional factor and oligosaccharides that are present in cereals and legumes used as weaning food. Adeyemi and Beckly (1986) reported that fermentation is able to solve some of the problems involved in the preparation of adequate weaning food for infant. It also reduces the phytic acid content by the action of enzyme phytases (produced during fermentation) which catalyses the conversion of phytate to inorganic orthophosphate. Tannin, protease and trypsin inhibitor content has also been reportedly reduced by fermentation (Haard, 1999; MacDonald *et al.*, 2012).

2.9 Plasmids

Plasmids are extra chromosomal DNA molecules. They are circular DNA molecules that replicate independently of the bacterial chromosome (Prescott *et al*., 1999, Wang *et al*., 2011). Plasmids are small, circular molecules of DNA containing genetic information. They contain about 2 percent of the genetic information of a cell and are separate from chromosomes. They are essential for the bacterium in that they may give it a selective advantage. Some plasmids determine the production of proteins that can kill other bacteria; others make bacteria resistant to antibiotics. Plasmids are extremely

valuable tools in the fields of molecular biology and genetics, specifically in the area of genetic engineering (Bruce, *et al*., 2002; Bulut *et al.*, 2005).

Plasmids play important roles in the lives of organisms that have them. They have proved invaluable to microbiologists and molecular geneticists in constructs and transferring new genetic combinations cloning gene (Prescott *et al*., 2008).

In general, bacterial plasmids can be classified into two groups on the basis of the number of genes and functions they carry. The larger plasmids are Deoxyribonucleic acid (DNA) molecules of around 100 kilobase (kb) pairs, which is sufficient to code for approximately 100 genes. There are usually a small number of copies of these plasmids per host chromosome, so that their replication must be precisely coordinated with the cell division cycle. The plasmids in the second group are smaller in size, about 6–10kb. These plasmids may harbor 6–10 genes and are usually present in multiple copies (10–20 per chromosome) (Bruce *et al*., 2002; Wang *et al.*, 2011).

Both linear and circular plasmids have been documented, but most known plasmids are either circular or linear which possess special structure or sequence at the ends to prevent their degradation and to permit their replication. They have few genes generally less than 30. Their genetic information is not essential to the host and all the cells that lack them usually function normally. However, many plasmids carry genes that confer a selective advantage to their hosts in certain environment (Prescott *et al*., 2008).

Plasmids are able to replicate autonomously. Single copy plasmids produce only one copy per host cell, some are able to integrate into the chromosome and are thus replicated with the chromosomes. Such plasmids are called **Episomes**. Plasmids are inherited stably during cell division, but are not always equally apportioned into daughter cells and sometimes are lost (Mark *et al.*, 1996; Bulut *et al.*, 2005).

They carry genes that confer a selective advantage on their host, such as resistance to heavy metals or resistance to naturally made antibiotics carried by other organisms. Alternatively, they may produce antibiotics that help the host to compete for food or space. For instance, genes produced by a plasmid will allow its host bacteria to grow even in the presence of competing bacteria or fungi that produce these antibiotics (Mark *et al.*, 1996).

Plasmids are also useful tools in research and biotechnology. Because of their ability to move genes from cell to cell; plasmids have become versatile tools for research and biotechnology. In the laboratory, researchers use plasmids to carry marker genes, allowing them to trace the plasmid's inheritance across host cells. Transferred or "cloned"

genes are used to produce a variety of important medical, agricultural, or environmental products that can be economically used by humans (Bruce *et al.*, 2002; Wang *et al.*,2012).

2.9.1 Types of Plasmids

Plasmids are sub-grouped into five main types based on phenotypic function, mode of existence, spread and function (Lordish *et al.*, 1999; Alcamo *et al*., 2000; Wang *et al*., 2011).

1. **Conjugate plasmids:** confer hair like structures called pill and can transfer copies of themselves to other bacteria during conjugation.
2. **F-factor**: it is responsible for the formation of fertility factor. F plasmids contain the F or fertility system required for conjugation (the transfer of genetic information between two cells). These are also known as episomes because, under some circumstances, they can integrate into the host chromosome and thereby promote the transfer of chromosomal DNA between bacterial cells.
3. **Resistance factor**: R-factors: They confer antibiotics resistance on cells that contain them. R plasmids carry genes encoding resistance to antibiotics. They also confer on their host the ability to produce antibacterial polypeptides called bacteriocins that are often lethal to closely related or other bacteria.
4. **Bacteria encoding plasmids:** are bacteria proteins that encode other bacteria. All bacterial genes are located on the plasmids.
5. **Virulence plasmids:** Encode factors that make the host more pathogenic. They confer pathogenicity on a host organism by the production of toxins or other virulence factors.

6 **Metabolic plasmids / Degradative or catabolic plasmids**

They carry the genes required to break down complex sugars into simple sugars. They also allow a host bacterium to metabolize normally undegradable or difficult compounds such as various pesticides and chemicals (Lordish *et al.*, 1999; Alcamo *et al*., 2000).

2.9.2 Plasmid Curing and Its Effects

Plasmid Curing: The loss of plasmid is called curing. It can occur spontaneously or be induced by treatments that inhibit plasmid replication but not host cell reproduction. Some commonly used curing treatments are acridine mutagens, ion and ionizing radiation, thyme starvation, antibiotics and growth above optimum temperature or extreme environmental conditions (Silhavy, 1994, Ogier *et al*., 2002).

Because of their ability to move genes from cell to cell, plasmids have become versatile tools for both research and biotechnology. In the laboratory, researchers use plasmids to carry marker genes, allowing them to trace the plasmid's inheritance across host cells. Transferred or "cloned" genes are used to produce a variety of important medical, agricultural, or environmental products that can be economically used by humans (Alcamo *et al.,* 2000; Bulut *et al.*, 2005).

2.10 Molecular Identification and Characterization of Bacteria

Since microorganisms were first isolated and grown in pure culture, microbiology laboratories have needed to characterize isolates so that they can be differentiated from one another. Schemes that can be used to describe the characteristics of a microbial isolate are essential in every branch of microbiology and their development and refinement have been constant (Lucke, 2000; Olaoye and Onilude, 2009). The advent of molecular biology in the 1980s contributed a set of powerful new tools that have helped microbiologists to detect the smallest variations within microbial species and even within individual strains (Olaoye and Onilude, 2009; http:www.ncib.nlm.nih.gov/pubmed3200828; http://www.lab-rech-associatives.com/pdf/utiliser%201a%).). This has added an entirely new dimension to a science that was in danger of becoming constrained by its reliance on traditional laboratory techniques.

In fact, the technology has progressed far beyond the level needed by most routine laboratories, where identifying the species of any isolate is likely to be sufficient. Distinguishing between different strains of the same species – typing – is more likely to be of value in a research laboratory. Nevertheless, methods and equipment designed to help with both species identification and typing are commercially available for a range of applications (Jahns *et al.,* 1995; Lucke, 2000).

The identification of a microbial isolate to species level only amounts to a partial characterization of the isolate, but is still a very useful piece of information. Knowing the species allows the laboratory access to the body of knowledge that exists about that species (Jahns *et al.,* 1995; Ogier *et al.,* 2002). For example, is it a known human or animal pathogen, or is it likely to multiply in product and cause spoilage. An identification of the species may also provide a clue as to the source of a contaminant (Jahns *et al.,* 1995; Ogier *et al.,* 2002). The number of recognized species of microorganisms has constantly increased – there are now more than 8,000 bacterial

species included in the *Approved Lists* – particularly since microbiologists acquired the ability to investigate the genetic relationships between different isolates (Amann *et al*., 1994; Ogier *et al.*, 2002). Despite this, it is still possible for routine microbiology laboratories to identify many isolates to genus, and often to species level using a remarkably small number of key tests (Amann *et al.*, 1994, Lucke, 2000).

Identification schemes using characteristics such as colony and cell morphology, Gram reaction and other staining characteristics, nutritional and physical requirements for growth, metabolic characteristics and pathogenicity factors have been developed and improved over many decades to a point where even small laboratories are able to identify isolates to species level using fairly simple traditional test procedures (Hammes *et al.*, 1992; Blot *et al.*, 1994; Ogier *et al.*, 2002).

Many of these tests, especially those related to metabolic characteristics, have been packaged into easy-to-use kits and even automated systems, equipped with internal databases, against which isolates can be compared to obtain an accurate identification (Hammes *et al.*, 1992; Blot *et al.*, 1994). Such kits and systems provide in most cases, a result within the same working day or next day, and achieve very significant savings in materials and technician time, largely replacing the need to perform manual tests on individual isolates (Blot *et al.*, 1994).The characteristics used in traditional microbial identification schemes are all observable aspects of the organism's structure and function. In other words they are phenotypic characteristics and are the products of gene expression (Guthier *et al.*, 1995; Lucke, 2000; http:www.ncib.nlm.nih.gov/pubmed3200828; http://www.lab-rech-associatives.com/pdf/utiliser%201a%).).

Recently, scientists look directly at the microbial genome itself and from this identify a species using its genotypic characteristics. Many bacterial species can now be identified by sequencing specific sections of ribosomal DNA – the 16S rRNA gene, this is most commonly used now , after amplification by PCR, and then comparing the results to sequences stored on a related database (Guthier *et al.*, 1995; Lucke ,2000). The commercially available system based on this technology is a valuable complementary tool to other routine identification technologies (Guthier *et al.*, 1995). However, identification based on the 16S rRNA gene is by no means infallible. Indeed as the sequence stretch analysed is a reduced section of the full genome and the variability of this marker is low. Furthermore, the accuracy of the system is dependent on the quality of the database against which the sequence is compared (Hammes *et al.*, 1992; Guthier *et*

al., 1995; Ogier *et al.,* 2002; http:www.ncib.nlm.nih.gov/pubmed3200828; http://www.lab-rech-associatives.com/pdf/utiliser%201a%).

2.10.1 Molecular characterization Techniques

2.10.1.1 Genotyping

The development of techniques for direct study of the microbial genome, notably amplification by PCR, has led to a relative explosion of published methods for typing microorganisms over the last 20 years (Gomez-luz *et al.,* 1996). Ideally one would sequence the entire genome of an isolate to provide definitive typing. This is technically feasible, but only available to those with several years to spare and an unlimited budget (Gomez-luz *et al.,* 1996; Bulut *et al.,* 2005). Therefore more practical, if slightly less discriminating, methods for analysing DNA extracted from microbial cells – often termed 'DNA fingerprinting' techniques – have been developed and some of the more widely used methods are outlined below as described in the websites listed below (http:www.ncib.nlm.nih.gov/pubmed3200828; http://www.lab-rech-associatives.com/pdf/utiliser%201a%).

1. **Multilocus sequence typing (MLST)** – sequencing 400-500 base pair fragments of DNA at seven different conserved genes allows small variations within a species to be detected. Quite time consuming and costly, but can be highly discriminatory if the genes are correctly chosen (Gomez-luz *et al.,* 1996; Ogier *et al.,* 2002).
2. **Pulsed-field gel electrophoresis (PFGE)** – a technique that allows the electrophoretic separation of low numbers of large DNA restriction fragments produced using restriction enzymes to generate a highly discriminatory genetic fingerprint. Widely used for the method of choice in the typing of human bacterial pathogens and the investigation of disease outbreaks. PFGE is relatively costly and requires at least three days to obtain a result. The degree of discrimination also depends on choice of restriction enzymes (Gomez-luz *et al.,* 1996.)
3. **Ribotyping** – this technique relies on the relative stability of the 16S and 23S rRNA genes coding for ribosomal RNA. The genes are cut using restriction enzymes and resulting DNA fragments separated by electrophoresis. The resulting fingerprint is visualised using fluorescent probes. Ribotyping has been developed into an automated system that is commercially available with dedicated databases. It is rapid (<24 hours to result), reproducible and works for a wide range of bacterial species, but is relatively costly in terms of equipment (Gomez-luz *et al.,* 1996).

4. **Repetitive sequence-based PCR (rep-PCR)** – bacterial and fungal genomes contain numerous non-coding, repetitive DNA sequences separating longer, single copy, sequences and their arrangement varies between strains. The rep-PCR technique relies on amplifying these repetitive sequences to produce amplicons of varying length that can be separated by electrophoresis giving a fingerprint comprised of bands that fluoresce at different intensities after binding with an intercalating dye. Rep-PCR has also been developed into a commercial typing system giving rapid results and using dedicated software to aid typing. The system is widely used for typing human pathogens (http://ww:rapid microbiology.comlopenlink php? flink=http%3A%2F%; www.foodsafetywatch.com_test_method_3133c_TM_B_Molecularidentification.php_13 05014710; Hammes *et al.*, 1992; Guthier *et al.*, 1995; Gomez-luz *et al.*, 1996 Ogier *et al.*, 2002).

2.10.2 Advantages of Molecular Characterization over the Conventional Methods

Recently, the molecular method of identification and characterization of micro organisms have been preferred over the classical ones which make use of the biochemical reactions and proteolytic activities of the organisms (Ogier *et al.*, 2002;Olaoye and Onilude,2009).The classical and conventional method of identification is slow, laborious, time consuming and may not be 100% specific and accurate. It is also problematic due to ambiguous biochemical or physiological traits (Sierra *et al.*, 1995). Workers like Sierra *et al.* (1995) and Bulut *et al.* (2005) reported that identification of LAB by phenotypic methods such as sugar fermentation may be uncertain and complicated owing to the increase in species that vary with few characters.

Furthermore, new species of LAB are continually being identified, making further identification necessary. There are also several assumptions in the phenotypic method of identification and these assumptions are subjective and are based on the analyst point of judgement .It therefore has so many limitations (Hugas *et al.*,1993; Bulut *et al.*, 2005; http:www.ncib.nlm.nih.gov/pubmed3200828 ;http://www.lab-rech-associatives.com/pdf/utiliser%201a%).

The molecular methods of identification however make use of the genetic composition of the organism in carrying out its identification (Hugas *et al.*, 1993). This is usually precise and accurate because no two organisms have the same gene or nucleotide sequence. It has also been used to simplify different identification methods because it is able to provide rapid, reliable, accurate and reproducible results that are also used to

confirm organisms that have been identified using phenotypic methods of identification and characterization earlier. It is also fast and does not waste time (Quere *et al.,* 1997; Lucke, 2000).

The development of molecular typing methods has offered the possibility of accelerating a great deal of bacterial identification which avoids so many biases that are related to the classical methods. The Polymerase chain reaction (PCR) has however provided a method to detect DNA sequences with high speed and sensitivity. This technique is emerging as a new tool in identifying and selecting bacteria with specific and desirable functions (Bulut *et al.*, 2005; Olaoye and Onilude, 2009). The method is rapid, inexpensive, and relatively easy way of producing a large number of copies of a specific DNA sequence for identification of bacteria (http:www.ncib.nlm.nih.gov/pubmed3200828; http://www.lab-rech-associatives.com/pdf/utiliser%201a%).

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CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Sample Collection

The samples used were *ogi* – made from Local varieties of white and yellow maize (*Zea mays*) and brown *ogi* from Sorghum (*Sorghum bicolor*). The cereals were purchased thrice at different locations including Bodija, Sango and Iwo Road area of Ibadan. Typed varieties of maize and sorghum named 'Ex-Kano', 'Samsorg 40' and 'Samsorg 41' were also obtained from the Institute of Agricultural Research and Training(IAR&T), Moor Plantation, Ibadan. They were all processed to *ogi* in the Laboratory using the traditional method of Banigo and Muller (1972). *Ogi* was also obtained from traditional sellers within Ibadan -white, yellow and sorghum *ogi* and used for comparative studies. The samples were collected in clean polythene bags and transported to the Laboratory. The flow chart for *ogi* production is shown below:

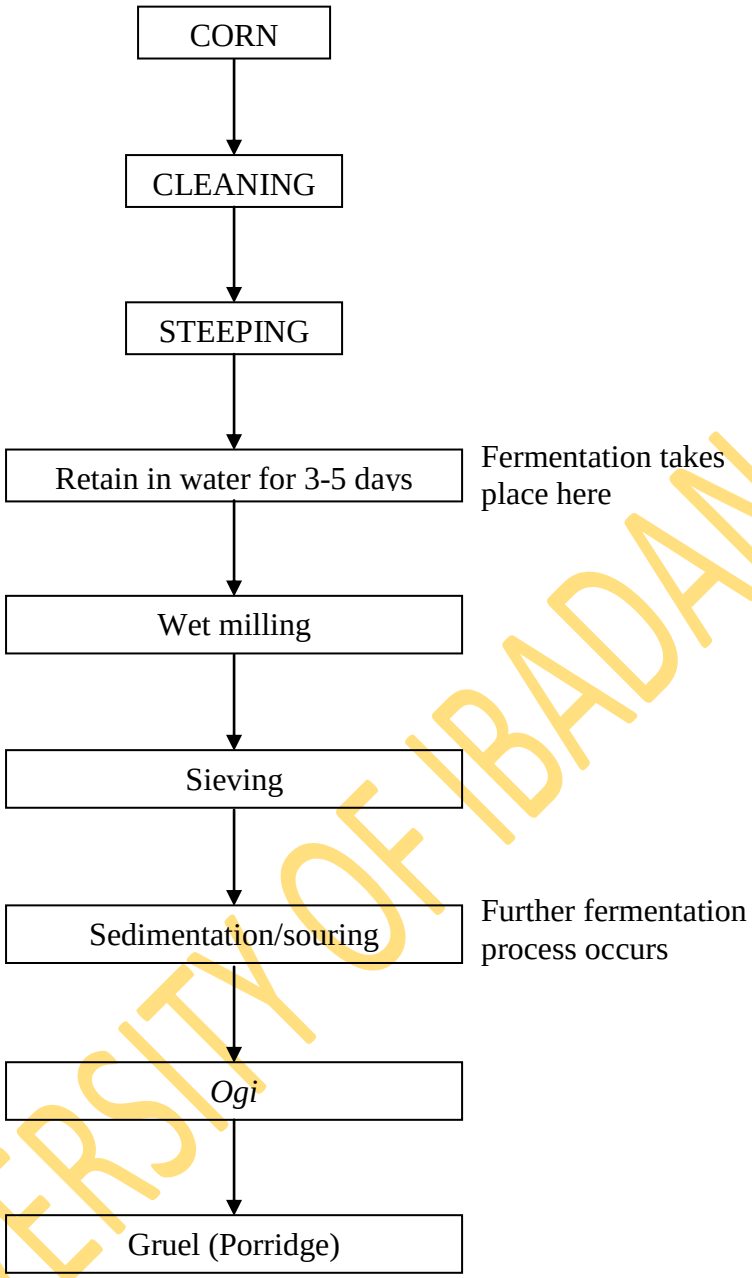

Figure 3.1: Flow Chart for *Ogi* Production (Banigo and Muller, 1972)

3.2 Isolation And Culture Methods

3.2.1 Isolation of Lactic Acid Bacteria

One gram each of the 9 samples listed above was subjected to ten-fold serial dilutions using the method of Harrigan and MacCance (1976). Each of the samples was mixed thoroughly with 9ml of sterile distilled water to give 10^{-1} dilution. 1ml of this was transferred into another 9ml of sterile distilled water with a sterile pipette in a sterile screw capped bottle to give 10^{-2}. This was repeated for 3 other screw-capped bottles that have been filled with 9ml sterile distilled water to give $10^{-3,}$ 10^{-4} and 10^{-5} dilutions respectively.

Isolation of organisms was done with the pour plate method (Harrigan and MaCance, 1976). With sterile pipette, aliquot of 1ml of the higher dilutions (10^{-4} and 10^{-5} dilutions) was pipetted into sterile Petri dishes and molten MRS agar cooled to 45^0C was poured. It was swirled gently for distribution of the inoculum in the agar, after solidification, incubated anaerobically in an anaerobic jar at 30^0C for 72 hours.

3.3 Identification of the Isolates

3.3.1 Morphological Characteristics

For proper identification of the isolates, the cultural, morphological, biochemical and physiological characterization including microscopic and macroscopic examinations of the various isolates were carried out according to Olukoya *et al.*, (1993a); Olsen *et al.* (1995).

3.3.1.1 Macroscopic Examination

Macroscopic observation of the different colonies as they appeared on MRS agar after incubation was done. The colour, smell, appearance, size, elevation, edges and degree of growth, surface and margin were also noted and recorded. Pure cultures were examined microscopically using high power objective with immersion oil.

3.3.1.2 Microscopic Examination

3.1.1 Gram's Staining

The pure culture of each isolate was stained as described by Norris and Ribbond (1976). A thin smear of the isolate was made on a clean slide and heat-fixed by passing it over a flame. 1ml of crystal violet was added to the smear and was allowed to stay for 60secs. The crystal violet was washed off under running tap water and it was then stained with Gram's iodine solution and left for another 60 seconds. The iodine was rinsed off by

flooding the slide with ethanol for decolourization to take place. Two drops of safranin was added as counter stain for 10secs and it was then rinsed with tap water. It was allowed to dry and observed using oil immersion objective lens. Gram positive bacteria were characterized by purple colouration while Gram negative cells were stained pink. The staining technique also showed the shapes and arrangement of the cells of the isolates (Olsen *et al*., 1995).

3.3.1.3 Preservation of Pure Cultures of LAB Isolates

Eighteen to twenty four hours old pure cultures of the isolates were aseptically transferred to prepared MRS slants. The organisms were preserved by over laying the slants with 12% (w/v) sterile glycerol after incubating for 48hrs at 30^0 C and was stored in the refrigerator. The preserved cultures were used for all the various tests that were carried out with the isolates (Olsen *et al*., 1995).

3.3.2 Biochemical Tests for characterisation of isolates

3.3.2.1 Catalase Test

This test was done to determine the presence of enzyme catalase possibly produced by the isolates. The enzyme catalase catalyses the breakdown of hydrogen-peroxides to release oxygen gas.

A twenty four hours old culture was used to make a smear on a clean slide. 3% hydrogen peroxide was then added to the culture on the slide. Production of a gas which was indicated by the presence of air bubbles on the slide indicates a positive reaction. A negative reaction shows no gas or bubbles.

$$2H_2O_2 \rightarrow \quad O_2 + 2H_2$$

(Seeley and van Demark, 1972)

3.3.2.2 Oxidase Test

This detects the presence or absence in bacteria of some oxides that will catalyse the transport of electrons between electron donors in the bacteria and a redox- dye.

Whatman filter paper (No 1) was placed in a sterile Petri dish and soaked with some drops of oxidase reagent (1% aqueous tetramethyl-p-phenylene diamine hydrogen chloride). A slide was then used to pick a colony of the organism and was used to touch the oxidase reagent on the filter paper.

The development of purple colouration within 5-10 seconds signifies an oxidase positive reaction. A delayed reaction or no colour change indicates a negative reaction (Seeley and Van Demark, 1972).

3.3.2.3 Methyl Red Test

This test was done to detect if the organisms can grow in a medium contain glucose and phosphate salt only. Glucose phosphate broth was prepared and sterilized and the organisms were inoculated into it after cooling. The test mixture was incubated for 48hrs after which 2-3 drops of methyl-red indicator was added to it. A positive result is indicated by the production of a bright red colour. A negative test shows yellow colour (Harrigan and McCone, 1976).

3.3.2.4 Voges Proskaeur Test

This test was done to test for the production of acetymethyl carbinol from glucose .Glucose phosphate broth was prepared and sterilized using membrane filteration and the test organisms were inoculated into it. 3ml of 5% alcoholic alpha-naphtol solution and 3ml of 40% KOH solution was added to it. Development of red colouration within 5mins indicates a positive result. A negative result shows yellow colouration. (Harrigan and McCance, 1976).

3.3.2.5 Indole Production Test

This test was done to show whether the organisms can degrade the amino acid tryptophan or not. Indole is a nitrogen containing compound. Tryptone broth (1%, w/v) was prepared and dispensed into screw-capped bottles and sterilized using membrane filteration. The test organisms were inoculated with and incubated for 48hrs. 3ml each of Kovac's reagent was then added to 6ml of the culture broth and mixed thoroughly, a reddening of the alcohol layer indicate indole production in the tube. A negative test remains yellow.

A ring of alcohol was also observed on the mixture of the Kovac's reagent and the culture. (Harrigan and McCance, 1976).

3.3.2.6 Homofermentative /Heterofermentative Test

The semi-solid medium of Gibson and Abd-El-Malek (1945) as modified by Stanier *et al.* (1964) was used. The medium was divided into two. The first consisted of 2.5g/litre yeast extract, 50.0g/litre glucose, 0.04g/litre $MnSO_4.7H_20$ and distilled water

with 1000ml as the basal medium. It was dispensed into screw capped bottles (5ml each) with sterile pipette and sterilized. This was then cooled and inoculated in 2 replicates. The second medium, sterile agar seal, consisted of reconstituted skimmed milk (80g/80ml) of water and nutrient agar 5.6g/19ml of distilled water. This was also sterilized and allowed to cool and the agar seal was then poured on the surface of each bottle. Production of gas was indicated by gas bubbles forcing of the agar seal up the tube. Uninoculated tubes served as control.

3.3.2.7 Nitrate Reduction Test

Peptone water was prepared (1%). 0.1% potassium nitrate was then added to make Nitrate-Peptone water. The test shows whether the organisms were able to reduce nitrate to nitrogen gas or not. Sterilization was done and 5ml each was dispensed into screw capped bottles with sterile pipette. Durham tube was also introduced into the bottles before sterilization. It was allowed to cool and the test organisms were inoculated into it. Uninoculated tubes served as control. It was incubated for 72hrs at 30^0C. Presence of gas in the Durham tube after incubation showed the production of nitrogen gas by the isolates (Payne, 1973). The presence of Nitrate was detected by adding 0.5ml of 0.1% sulphalinic acid in 5N acetic acid to the various screw capped bottles.

A positive result was shown by the development of red colouration as a ring on top of the broth and gas in the Durham tubes. A negative result remains yellow. (Harrigan and McCance, 1976).

3.3.2.8 Growth in 4% NaCl Broth

MRS broth containing 4% (w/v) NaCl was prepared and sterilized at 121^0C for 15mins. 20ml of the broth was aseptically dispensed into sterile screw capped vials. After cooling, the tubes were inoculated with the test organisms and incubated for 48hrs. Increased turbidity of the medium was recorded as positive. A negative result shows no turbidity. Uninoculated tubes served as control (Schillinger and Luke, 1987).

3.3.2.9 Production Of Ammonia From Arginine

This shows whether the organism produce ammonia from the essential amino acid-arginine. Modified MRS broth into which arginine had been added and glucose and meat extract excluded was used for this test. 0.3% arginine and 0.2% sodium citrate was

used in place of triamnonium citrate. The medium was dispensed into screw-capped bottles (10ml each), sterilized and the test organisms inoculated into it after it had cooled down. This was then incubated at 30^0C for 72hrs after which some few drops of Nessler's reagent were added to the various tubes.

A positive reaction is indicated by the development of deep yellow colour while a negative result does not show this (Harrigan and McCance, 1976).

3.3.2.10 Citrate Utilization

Simmons Citrate medium was prepared and dispensed into screw capped bottles and sterilized. The contents of each tube were allowed to cool and the test organisms inoculated into it. It was then incubated at 30^0C for 48hrs after which the colour change was observed.

A positive result was shown by a change in colour of the medium from green to blue while a negative result remains unchanged (green). The test shows whether the organisms are able to utilize citrate or not (Nester *et al.*, 1985).

3.3.2.11 Motility Test

This test shows whether the organism is motile or not. The "Hanging Drop Technique" which involves the use of immersion oil around the edge of the depression of a cavity slide was used. A drop of the test organisms was introduced into the depression and covered with cover slip. This was then inverted over the cover slip such that the culture drop is in the centre of the slide depression and examined under x10 and x40 objectives of a light microscope.

A positive test is shown under the microscope by the presence of flagella on the organisms which it uses for movement while a negative test does not show movement at all (Seeley and Van Demark, 1972).

3.3.2.12 Gelatin Hydrolysis

10% gelatin broth was prepared and 5ml each was dispensed into screw capped bottles and sterilized by autoclaving at 121^0C for 10mins. After cooling, it was inoculated with the test organisms and incubated for 72hrs at 30^0C. It was then transferred into the refrigerator and left overnight. Uninoculated tubes served as control.

A positive result was shown by the gelling of the gelatin broth while a negative result did not gel. This shows whether the organisms are able to utilize/breakdown gelatin

or not. By gelling of the broth, it means the gelatin has not been utilized by the organisms (Harrigan and McCance, 1976).

3.3.2.13 Casein Hydrolysis

This test shows that the organisms are able to utilize or hydrolyse casein or not .1% skimmed milk was added to nutrient agar and sterilized. The pour-plate method of inoculation was then used to inoculate the organisms on the skimmed-milk nutrient agar plate. This was incubated at 30^0C for 72hrs. Uninoculated tubes served as control.

A positive result is indicated by growth of the organisms on the nutrient agar plates. A negative result shows no growth (Harrigan and McCance, 1976).

3.3.2.14 Starch Hydrolysis

Starch agar plate prepared by substituting glucose with soluble starch in the appropriate amount in the modified MRS agar was used. The various test isolates were then inoculated by streaking on each of the plates and incubated for 3 days at 30^0C. Amylase production was tested for by flooding the plate with diluted Gram's iodine solution.

The development of blue-black colouration on the starch agar plate and presence of a clear zone along the line of streak shows amylase production. This shows that the amylase produced has hydrolyzed the starch in the medium. A negative test will not show this (Seeley and Van Demark, 1972).

3.3.2.15 Sugar Fermentation

a) Fermentation of isolates in a complex medium

Modified MRS medium without meat extract and D-glucose was prepared. The D-glucose component was substituted with quantity (w/v) of the test sugars: raffinose, sucrose, lactose. Fermentation was allowed to take place by incubating the broth at 30^0C for 5 days. The growth of the organisms which was indicated by increase in turbidity was monitored by taking their absorbances at different time intervals from 0-120hrs using a Cecil 2031 spectrophotometer was used to detect. The concentration of carbon sources was also varied. Uninoculated tubes served as control (Gibson and Abd-el-Malek, 1945).

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b) **Sugar Fermentation**

Modified MRS medium without meat extract and D-glucose was prepared. The D-glucose component was substituted with equivalent quantity (w/v) of the test sugars. The various sugars used were xylose, rhamnose, trehalose, raffinose, sucrose, lactose, maltose, galactose, fructose, arabinose, mannose, dulcitol, manitol and inositol. 0.0016% bromocresol purple was added as an indicator for acid production by the isolates as signified by a change of colour from purple to yellow. This was then dispensed into screw capped bottles (10ml each). Durham tube was also introduced into in an inverted manner to detect gas production. The sugars were sterilized by membrane filteration so as not to denature the sugars. The tubes were inoculated with the test organisms and incubated at 30^0C for 5-7 days. Uninoculated tubes served as control.

A change of colour from purple to yellow indicated acid production while gas production was also noticed in the Durham's tube. No change in colour was observed for negative tests (Harrigan and McCance, 1976).

3.4 Physiological Studies

3.4.1 Growth at Different Temperatures

The test was done to determine the best temperature for the growth and metabolism of the isolates as indicated by increased turbidity. MRS broth was prepared and dispensed into series of screw-capped bottles and sterilized. It was allowed to cool and the test organisms inoculated into it. These were incubated at different temperatures 15^0C, 20^0C, 30^0C, 40^0C, 45^0C 50^0C, 60^0C, 70^0C, 80^0C, for 72hrs after which Cecil 2031 spectrophotometer was used to detect increase in turbidity. The concentration of carbon sources was also varied. Uninoculated tubes served as control (Gibson and Abd-el-Malek, 1945).

3.4.2 Growth at Different pH

This test was done to determine the best pH for the growth of the isolates as indicated by the increased turbidity .MRS broth was prepared and the pH was adjusted using 0.1m phosphate buffer to 3.0, 3.9, 4.0, 5.0, 6.0, 7.0, and 9.2. It was then dispensed into screw capped bottles and sterilized in the autoclave at 121^0C for 15mins. After cooling, the various test isolates were standardized using MacFarland standard to 3.1 x 10^3 cfu/ml with optical density adjusted to 0.5. With a sterile pipette, 1ml of the standardized was isolates inoculated into it and incubated at 30^0C for 48hrs. Growth was

detected by increased turbidity using Cecil 2031 spectrophotometer. Uninoculated tube served as control. The concentration of carbon sources was also varied (Schillinger and Lucke, 1989).

3.4.3 Growth at Different Concentration of Carbon Sources

This test was done to detect the best concentration of the sugars that favour growth and metabolism of the isolates. MRS broth was prepared and varying concentration of carbon sources (mM concentration) - Glucose, Lactose and Raffinose were used. The concentration used was within the range of 0.1-2.0mg/ml and 10ml each was dispensed into screw-capped bottles. This was sterilized by membrane filteration, allowed to cool and the organisms inoculated into it. Uninoculated tubes with the carbon sources served as control. It was incubated at 30^0C for 48hrs. Growth was detected by increased turbidity using Cecil 2031 Spectrophotometer (Gibson and Abd-el-Malek, 1945).

3.4.4 Growth at Different Concentration of Nitrogen Sources

This test was done to detect the best concentration of nitrogen sources that favours the growth and metabolism of the various isolates. MRS broth was prepared and varying concentration of Nitrogen sources in form of its salt (mM concentration) was used in the presence of Lactose and Raffinose as carbon sources. The Nitrogen sources used include Peptone, Ammonium chloride and Sodium nitrate. The concentration used was within the range 0.1-1.5mg/ml. 10ml each was dispensed into screw-capped bottles. This was sterilized, allowed to cool and the test organisms inoculated into the bottles. It was then incubated at 30^0C for 48hrs after which growth was observed. Uninoculated tubes served as control. Growth was detected by increased turbidity using Cecil 2031 Spectrophotometer (Gibson and Abd-el-malek, 1945).

3.4.5 Growth at Different Anion Concentration

This test was done to detect the best concentration of the anion that favour growth and metabolism of the isolates MRS broth was prepared and varying concentration of anions in form of its salt (mM concentration) was used in the presence of Glucose, Lactose and Raffinose as carbon sources. The anions used were Triammonium citrate, Magnesium chloride and Sodium chloride. The concentration used was within the range of 0.1-2.0mg/ml, 10ml each was dispensed into screw capped bottles. This was sterilized,

allowed to cool and the organisms inoculated into it. Uninoculated tubes served as control. It was incubated at 30^0C for 48hrs. Growth was detected by increased turbidity using Cecil 2031 spectrophotometer (Gibson and Abd-el-Malek, 1945).

3.4.6 Growth at Different Cation Concentration

This test was done to detect the best concentration of cation that favours the growth and metabolism of the various isolates. MRS broth was prepared and varying concentration of cations in form of its sugar (mM concentration) was used in the presence of Glucose, Lactose and Raffinose as carbon sources. The cations used were magnesium sulphate, ferrous sulphate and zinc sulphate. The concentration used was within the range 0.1-1.5mg/ml. 10ml each was dispensed into screw-capped bottles. This was sterilized, allowed to cool and the test organisms inoculated into the bottles. It was then incubated at 30^0C for 48hrs after which growth was observed. Uninoculated tubes served as control. Growth was detected by increased turbidity using Cecil 2031 Spectrophotometer (Gibson and Abd--malek, 1945).

3.5 Enzymes Production by the Isolates

3.5.1 Inoculum Preparation

The LAB isolates that were used for the various tests were prepared by inoculating a colony from a 24hr old LAB streaked on a plate into a sterile 9ml MRS broth. This was incubated for 24hrs at 30^0C.

3.5.2 Determination of Inoculum size

The LAB isolates that were used were standardized according to MacFarland standard using $BaCl_2$ and HCl at the right proportion. The culture supernatant was also brought to the same optical density (OD) of 0.500, using sterile MRS broth (Olutiola *et al.*, 1993).

3.5.3 Medium Preparation, Inoculation and incubation

The medium used was MRS-Starch broth in which the glucose had been substituted with equivalent amount (w/v) of soluble starch. The medium was dissolved and homogenized in a water bath (Uniscope 801A Model, England) after which it was dispensed into Erlenmeyer flasks in aliquots of 250ml, plugged with non-absorbent cotton-wool and aluminium foil. It was sterilized using membrane filteration. With a sterile pipette, 10ml of the standardized isolates were inoculated into it and incubated at 30^0C on a shaker for 48hrs.

3.5.4 Extraction of Enzymes

The culture–broth was centrifuged at 10,000 rpm for 15mins using refrigerated centrifuge. The cell-free culture supernatant was labelled as the crude enzyme while the sediment (microbial cells) was stored in the refrigerator for further use. This was then assayed for amylase, invertase and mellibiase production and activity (Lealem and Gashe, 1994).

3.5.5 Amylase Assay

Amylase activities of the organisms were determined using DNSA reagent method of Bernfeld (1955) as modified by Giraud *et al.* (1991). 1ml of culture supernatant was added to 1ml of the substrate containing 1.2% (w/v) soluble starch in 0.1M phosphate buffer, pH 6.0. The enzyme-substrate mixture was incubated at 30^0C for 10minutes. The reaction was stopped by the addition of 5M NaOH. The amount of reducing sugar thus produced was estimated according to Bernfeld method (1955) as modified by Giraud *et al.* (1991) with 3, 5,-Dinitrosalicylic Acid (DNSA). 1ml of the DNSA reagent was added to the filtrate-substrate-reaction mixture above which then was heated in boiling water at 100^0C for 10mins and cooled with distilled water. The absorbance was measured at 540nm using Cecil 2031 spectrophotometer, England.

1ml of uninoculated blank similarly treated was used to set the spectrophotometer at zero (Bernfeld 1955; Giraud *et al*., 1991).

3.5.6 Invertase Assay

Invertase activity of the organisms was determined using DNSA reagent method of Bernfeld (1955) as modified by Giraud *et al.* (1991). 1ml of culture supernatant was added to 1ml of a solution containing 1.2% sucrose in 0.1M phosphate buffer, pH 6.0. The enzyme–substrate (sucrose)-reaction mixture was incubated at 30^0C for 10mins.

The reaction was stopped by the addition of 5M NaOH. The amount of reducing sugar produced was estimated according to Bernfeld method (1955) as modified by Giraud *et al.* (1991) with 3,5-Dinitrosalicyclic acid which was prepared as stated in Appendix 3.2 . One ml of the DNSA reagent was then added to the filtrate-sucrose reaction mixture above and was heated in a boiling water bath at 100^0C for 10mins and cooled with distilled water. The absorbance was measured at 540nm using Cecil 2031 spectrophotometer, England. 1ml of uninoculated blank similarly treated was used to set

the spectrophotometer at zero. The amount of Invertase produced was read and recorded as being equivalent to the reducing sugar produced (Bernfeld, 1955; Giraud *et al.*, 1991).

3.5.7 Mellibiase Assay

Mellibiase activities of the organisms were determined using DNSA reagent method of Bernfeld (1955) as modified by Giraud *et al.* (1991). 1ml of culture supernatant was added to 1ml of a solution containing 1.2% lactose in 0.1M phosphate buffer, pH 6.0. The enzyme–substrate (lactose)-reaction mixture was incubated at 30^0C for 10mins.

The reaction was stopped by the addition of 5M NaOH. The amount of reducing sugar thus produced was estimated according to Bernfeld method (1955) with 3, 5-Dinitrosalicyclic acid which was prepared as stated in the Appendix 3.2. 1ml of the DNSA reagent was then added to the filtrate-sucrose reaction mixture above and was heated in a boiling water bath at 100^0C for 10mins. It was then cooled with distilled water. The absorbance was measured at 540nm using Cecil 2031 Spectrophotometer. 1ml of uninoculated blank similarly treated was used to set the Spectrophotometer at zero. The amount of Invertase produced was read separately and recorded while that of Mellibiase was read as being equivalent to the reducing sugar produced (Bernfeld, 1955; Giraud *et al.*, 1991)

One amylase unit is the amount of enzyme in 1ml of the filtrate, which releases 1mg of reducing sugar as glucose from a 1% starch solution in 1hr at 30^0C.

One Invertase unit is the amount of enzyme in 1ml of the filtrate which releases 1mg of reducing sugar as glucose from a 1% sucrose solution in 1hr at 30^0C.

One Mellibiase unit is the amount of enzyme in 1ml of the filtrate, which releases 1mg of reducing sugar as glucose from a 1% lactose solution in 1hr at 30^0C.

3.5.8 Galactosidase Production and Growth

MRS medium was prepared in which the glucose was substituted with equivalent quantity of Galactose. This was then dispensed into screw capped bottles in 10ml aliquots and sterilized using the autoclave (Progen Europe 13060 Top Loading Model). After cooling, the standardized organisms of inoculum size 3.1 x 10^3 cfu/ml were inoculated into it and incubated at 30^0C for 24hrs. Growth was observed through increased or reduced turbidity using Cecil 2031 spectrophotometer, England at 540nm. This test was

done to detect whether the organisms can utilise these carbon sources for growth and metabolism. Uninoculated tubes served as control (Giraud *et al.* 1991).

3.5.9 Effect of different Carbon sources on Growth and alpha-galactosidase production by the isolates

This test was done to detect whether the organisms can utilise different carbon sources for growth and metabolite production. MRS medium in which glucose was substituted with equimolar quantities of Glucose, Lactose and Raffinose was prepared. The culture fluids were analysed for growth while the culture fluids from the above were centrifuged at 10,000 rpm for 15min in each case for enzyme production (Lealem and Gashe, 1994). The media prepared was dispensed into screw capped bottles in 10ml aliquots and were sterilized using the autoclave (Progen Europe 13060 Top Loading). After cooling, the standardized organisms of inoculum size 3.1 x 10^3 cfu/ml were inoculated into it and incubated at 30^0C for 24hrs. Growth was observed through increased turbidity using Cecil 2031 spectrophotometer, England at 540nm. The cell-free supernatant fluid served as the source of crude enzyme. This was then asassayed for alpha- galactosidase production by the method as described previously. Uninoculated tubes served as control (Bernfeld, 1955; Lealem and Gashe, 1994).

3.5.10 Assay of Alpha-Galactosidase and Characterization

Alpha galactosidase activity was determined using the method of Mital *et al.*(1973) as modified by Hassan and Durr (1974). The assay medium consisted of 200µl of 100mM sodium acetate buffer, pH 5.0, 2.5ml of 2mM PNP- alpha Gal solution and 0.5ml of enzyme preparation. The assay was carried out for 15min at 50^0C and stopped by the addition of 1ml of 0.5M sodium carbonate. The amount of Para – nitrophenyl- α-D-galactopyranoside (PNP-α -G) released was determined at 410nm by taking the absorbance. Undiluted mixture treated in the same way was used to set the spectrophotometer to zero.

The materials used were

- ONPG – O – nitro phenyl – α-D-galactopyranoside
- Sodium acetate buffer, pH 5.0
- 2.5ml PNPG

- 0.5ml enzyme
- Reaction was stopped by the addition of 1ml of 0.5M sodium carbonate
- Amount of ONPG released was determined by taking the absorbance at 410mm.

*****One unit of enzyme activity was defined as the number of macromolecules of p-nitrophenyl liberated from PNP-G per ml of cells as read from a standard curve.

3.5.11 Assay of Beta- Galactosidase

Beta- galactosidase activity was determined using the method of Mital *et al.*(1973) as modified by Hassan and Durr (1974).The assay medium consisted of 200µl of 100mM sodium acetate buffer pH 5.0, 2.5ml of 2mM PNP- alpha Gal solution and 0.5ml of enzyme preparation. The assay was carried out for 15min at 50^0C and stopped by the addition of 1ml of 0.5M sodium carbonate.

The amount of Para – nitrophenyl- beta- D-galactopyranoside (PNP-beta -G) released was determined at 410nm by taking the absorbance. The undiluted mixture treated in the same way was used to set the spectrophotometer to zero.

Materials used were:

- ONPG – O – nitrophenyl – beta-D-galactopyranoside
- Sodium acetate buffer, pH 5.0
- 2.5ml ONPG
- 0.5ml enzyme

  Reaction was stopped by the addition of 1ml of 0.5M sodium carbonate
- Amount of ONPG released was determined by taking the absorbance at 410mm.
- One unit of enzyme activity was defined as the number of micromolecules of p-nitrophenyl liberated from PNP-G per ml of cells as read from a standard curve.

3.6 Determination of Reducing Sugar

1ml of the enzyme filtrate was added to 1ml of the standard starch substrate (1.2% soluble starch in 0.1M phosphate buffer at pH 6.0). This was incubated at 30^0C for 5 minutes. The reducing sugar produced was determined by adding 2ml of DNSA reagent. This was heated in boiling water bath at 100^0C for 5 minutes and cooled with tap water after which 20ml distilled water was added. The absorbance of the resultant solute was determined at 540nm with the aid of Cecil 2031 spectrophotometer, England. 1ml of the

inoculated medium similarly treated was used to set the transmittance at zero. The amount of sugar produced was determined from a standard curve obtained by recording the absorbance of increasing concentration of aqueous solution of maltose ranging from 0.2 – 2.0mg/ml. The result was then expressed as amylase unit (Bernfeld, 1955; Giraud *et al.*, 1991).

3.7 Determination of Lactic Acid, Hydrogen Peroxide and Diacetyl

3.7. 1 Determination of Lactic Acid Produced By the Isolates

Estimation of lactic acid was determined by titration of 25ml of 24 hr old broth cultures of the test organisms with 0.1N NaOH. 1ml of phenolphthalein was added as indicator. NaOH was then added slowly to the sample until a pink colour appeared. Each ml of 0.1N NaOH is equivalent to 90.08mg of lactic acid, as stated in A.O.A.C. (1990) and reported by Sanni *et al.* (1995).

$$\text{Titratable acidity of lactic acid} = \frac{\text{ml NaOH x NNaOH X M.E x 100}}{\text{Volume of Sample used}}$$

ml NaOH	=	Volume of NaOH used
N NaOH	=	Normality of NaOH
M.E	=	Equivalent factor = 90.08mg

3.7.2 Determination of Diacetyl Produced By the Isolates

The amount of diacetyl produced by the organisms was estimated by measuring 25ml of the broth cultures of the test isolates (24hrs) into conical flasks and 7.5ml hydroxyl amine solution was used for residual titration. The flasks were titrated with 0.1N HCl to a green-yellow end-point using bromophenol blue as indicator. The equivalent factor of HCl to diacetyl is 21.52mg as stated in the A.O.A.C and reported by Sanni *et al.* (1995).

$$AK = \frac{(b - s)(100 - e)}{w}$$

AK	=	percentage of diacetyl
b	=	no. of ml of 0.1N HCl consumed in titration of the sample
e	=	Equivalence factor = 21.52mg
w	=	Volume of sample
s	=	No. of ml of 0.1N HCl condensed in titration of residue sample.

3.7.3 Determination of Hydrogen Peroxide Produced By the Isolates

20ml of diluted H_2SO_4 was added to 25ml of the broth cultures of the test organism (24hrs). Titration was carried out with 0.1N potassium permanganate. Each ml is equivalent to 1.70mg of Hydrogen Peroxide and decolourization of the sample was regarded as end point (A.O.A.C, 1990).

Hydrogen Peroxide Concentration = $\frac{\text{ml } KMnO_4 \text{ x N } KMnO_4 \text{ X M.E. x 100}}{\text{ml } H_2SO_4 \text{ x Volume of sample used}}$

ml $KMnO_4$ = Volume of $KMnO_4$ used

$NKMnO_4$ = Normality of $KMnO_4$

mlH_2SO_4 = Volume of H_2SO_4 added

M.E = Equivalence factor = 1.701mg

3.8 Preparation of Processed Samples of Soybeans

3.8.1 Sterilization of Samples

All the samples of soybeans used for this analysis were first surface- sterilized by soaking in 1% Sodium hypochlorite solution for 5 mins. It was rinsed several times with distilled water and allowed to dry in an oven at 50^0C before further processing.

3.8.2 Preparation of Samples

RAW: 1kg of soybeans was used at each time for the processing. The samples were picked manually to remove the dirts. It was then milled into powder, sieved with a fine sieve to pass through 0.5mm diameter sieve and stored in air tight container for further use.

COOKED: One kilogram of soybeans was added to distilled water (1:5,w/v) and cooked at 80^0C on a hotplate. In all the cooking process, the level of cooking water was kept constant by the addition of boiling distilled water. It was then dehulled and washed several times with distilled water to remove the seed coat. It was drained and later dried in the oven at 50^0C. This was then milled into powder and sieved with a fine sieve. It was kept in airtight container for further use.

ROASTED: One kilogram of soybeans was roasted in the oven at 100^0C, dehulled to remove the seed coat and milled into powder. It was then sieved with a fine-sieve into fine powder. It was then stored in airtight container until further use.

3.9 Proximate Analysis

3.9.1 Determination of Moisture Content

Moisture content determination was carried out using the air oven method. Crucibles were washed and dried in an oven. They were allowed to cool in the desiccators and their weights were noted. A known weight of each sample were then transferred into the crucibles and dried at a temperature between 103^0-105^0C. The dried samples were cooled in a desiccators and the weight noted. They were later returned to the oven and the process continued until constant weights were obtained.

Calculation

$$\% \text{ Moisture content} = \frac{\text{Weight Loss X 100}}{\text{Weight of Sample}}$$

(AOAC, 1996)

3.9.2 Determination of Ash content

A known quantity of finely ground sample was measured into clean, dried previously weighed crucible with lid (W_1). The sample was ignited over a low flame to char the organic matter with lid removed. The crucible was then placed in muffle furnace at 600^{o}C for 6h until it ashed completely. It was then transferred directly to desiccators, cooled and weighed immediately (W_2).

$$\text{Percentage Ash} = \frac{W_2 - W_1 \quad \text{X } 100}{\text{Weight of Sample}}$$

(AOAC, 1996)

3.9.3 Determination of Crude Fat

The soxhlets extraction method (AOAC, 1996) was used. This method could only give the approximate fat content in a sample because all the substances soluble in chosen solvent (Petroleum ether, 40^0C – 60^0C boiling range) were extracted from the sample.

A known quantity of sample was measured into a weighed filter paper and folded neatly. This was put inside pre-weighed thimble (W_1). The thimble with the sample (W_2) was inserted into the soxhlets apparatus and extraction under reflux was carried out with petroleum ether (40^0C – 60^0C boiling range) for 6h. At the end of extraction, the thimble was dried in the oven for about 30 minutes at 100^0 C to evaporate the solvent and thimble was cooled in a desiccator and later weighed (W_3).

The fat extracted from a given quantity of sample was then calculated:

$$\% \text{ Fat (w/w)} = \frac{\text{Loss in Weight of sample X 100}}{\text{Original Weight of Sample}}$$

$$= \frac{W_2 - W_3 \text{ X } 100}{W_2 - W_1}$$

(AOAC, 1996)

3.9.4 Determination of Protein

The crude protein content was determined using micro Kjeldahl method as described in AOAC (1996). 0.2077g of sample was weighed into a long necked Kjeldahl flask. 1 tablet of Kjeldahl catalyst was added to the sample in the flask with 25cm^3 of conc. H_2SO_4. The flask was swirled, gently clamped in an inclined position and heated with electricity in a fume cupboard. The heating continued until a clear solution was obtained. The clear solution was cooled, poured into a 100cm^3 volumetric flask and made up to mark with distilled water 10ml of the resulting mixture was measured into the distillation set through the funnel. 5 cm^3 of boric acid was pipetted into a 100-cm^3 conical flask and placed at the receiving end of the distillatory. The conical flask was placed such that the delivery tube dipped completely into the boric acid inside the flask. 40% NaOH was used to liberate ammonia from the digest under alkaline condition. During the distillation, 2 drops of methyl orange were always added to the round bottom flask containing the digested sample before 40% NaOH was added.

As soon as the contents became alkaline, the red colour changed to yellow showing NaOH to be in excess. Steam was then generated into the distillation set using a steam chest. The liberated ammonia was trapped in the boric acid solution and about 50 cm^3 of the solution collected into a conical flask. The solution in the flask was titrated against 0.1M HC1 until the first permanent colour change was observed.

A blank sample was taken through the sample procedure and the titre value for the blank was used to correct the titre for samples.

$$\% \text{ N} = \frac{\text{Molarity of HC1 X (Sample titre – Blank titre) X 0.014 X df X 100}}{\text{Weight of sample used.}}$$

df= dilution factor

% N was converted to the percentage crude protein by multiplying by 6.25

(AOAC, 1996).

3.9.5 Determination of Crude Fibre

Two hundred ml (200ml) freshly prepared 1.25% H_2SO_4 was added to a known weight of the residue obtained from fat extraction and this was brought to quick boil. Boiling was continued for 30 minutes. The mixture was filtered and residue washed several times with water until it was free from acid. The residue was transferred into a digestion flask, 50ml of 1.25% NaOH was added and brought to boiling point quickly. Boiling was continued for 30 minutes. The mixture was filtered and residue washed free of alkali. The residue was then washed with methylated spirit, thrice with petroleum ether using small quantities. It was allowed to properly drain and the residue was transferred to a silica dish (previously ignited at 600^0 C and cooled). The dish and its content were dried to constant weight at 105^0 C. The organic matter of the residue was burnt by igniting for 30 minutes in a muffle furnace at 600^0 C. The residue was cooled and weighed. The loss on ignition was recorded as crude fibre (AOAC, 1996).

3.9.6 Determination of Carbohydrate

The carbohydrate content was calculated by difference.

% CHO = 100 - (Sum of the percentages of moisture, ash, fat, protein and crude fibre) (AOAC, 1996)

3.9.7 Determination of Ascorbic Acid

Vitamin C Volumetric Determination

Two grams of the sample was macerated in a mortar and filtered through a nylon cloth with distilled water. 25ml of the filterate was pipetted into a 50 ml flask and 12 ml of 20% Meta phosphoric acid, diluted to 50 ml with water was added. 10 ml of this solution was pipetted into a small flask and titrated with the 2:6 Dichlorophenol Indophenol solutions. Vitamin C was calculated as mg per 100 ml of juice (Pearson, 1991).

3.9.8 Determination of B Vitamins (Riboflavin and Thiamine)

1) Riboflavin

0.5g of the sample was weighed and 30ml 0f 30% HCl and Dichloroethane (ratio1:1) was added. 50ml Ammonium Hydroxide solution was added. It was filtered

using Whatman No1 filter paper. The absorbance was read on a spectrophotometer (Spectronic 20 model, England) at 415nm.
(AACC, 1990).

2) Thiamine

One gram of the sample was weighed and 50ml of 50% Methanol and 50ml of 17% Sodium Carbonate (ratio 1:1) was added. This represented the extract Sample was then filtered using Whatman filter paper No 1. To the filtrate, Folins-Denis Reagent was added. It was allowed to cool until a bluish colour was seen. The absorbance was read on a spectrophotometer (Spectronic 20 model, England) at a wavelength of 415nm.
(AACC, 1990).

3.9.9 Determination of Niacin

The niacin was extracted from the soybeans by autoclaving the sample with aqueous calcium hydroxide. The colorimetric method which requires the use of cyanogens bromide was used. Standards were prepared first. Soybeans samples of approximately 1 g were then mixed in centrifuge tubes with 0.75g calcium hydroxide and 20 ml deionised water. Each mixture was thoroughly mixed and heated in an autoclave for 30 min at 121^0C with the centrifuge tube cap loosened. The mixture was diluted to approximately 50 ml with water, mixed thoroughly and allowed to cool. The final volume was adjusted to 50 ml; the mixture was then mixed and centrifuged at 0^0C at 2500 rpm for 15 minutes. A 15-ml sample of the supernatant was adjusted to pH 7 with aqueous oxalic acid (10%) with 1% acid for the final adjustment) and made to 25 ml with water in a vial. The resultant suspension was centrifuged at 2500 rpm for10 minutes to precipitate the calcium oxalate.
(AACC, 1990).

3.9.10 Determination of Iron, Calcium and Phosphate in Food Samples

(Dry Digestion Method (Ashing))

0.2g of the sample was weighed into a clean crucible. The organic content was burnt off on an open flame. It was transferred into a muffle furnace and allowed to ash for 6hrs at 600^0C until the ash turned to white completely. This was washed with 10ml 0.1N HCl into a 100ml volumetric flask. It was warmed on a heater for few seconds to

avoid frothing. Filtration was done with Whatman No 42 (ashless) and was filtered into another 100ml flask.

Distilled water was added to the filtrate, to make it up to 100ml. This was stored as the stock for mineral determination.

Using an Atomic Absorption Spectrophotometer (AAS), the resonances of the different elements were taken.

Fe --set the AAS to 248nm

Ca -- set the AAS to 285 nm

$P0_4^{2-}$ --set the AAS to315 nm

The results were obtained from the spectrophotometer in meter reading. The calculation was done with the dilution factor.

$$\text{Dilution factor (df)} = \frac{\text{Total volume of the Extract}}{\text{Weight of the Sample}}$$

$$\text{Fe and Mg content of the Sample} = \text{MR X slope X df} = \text{ppms} = \frac{\%}{10{,}000}$$

where MR = Meter Reading

Slope = standard which is usually constant =1

ppms = parts per million sample

(AACC, 1990).

3.9.11 Determination of Total Sugar

Total sugar of the soybeans flour samples were determined using the procedure described by AOAC (1990) which involves the use of spectronic 20, England spectrophotometer. 0.025g flour was weighed into a centrifuge tube, and then the powder was wetted with 1.0 ml of ethanol. 2.0 ml of distilled water and 10 ml hot ethanol was added into the flour. The slurry was centrifuged for 10 minutes at 2,000 rpm and the supernatant was decanted into a test tube and was made up to 20 ml. For the colour development, an aliquot of 1 ml of extract was used for assay. 0.8 ml of distilled water, 0.5 ml of 5% phenol and 2.50 ml of concentrated H_2SO_4 was added. The solution was

allowed to cool and the absorbance was read at 490nm.The glucose standard curve was plotted.

The free sugar was calculated as

$$\% \text{ Sugar} = \frac{(A - I) \times D.F \times V \times 100}{B \times W \times 10^6}$$

Where A = Absorbance of sample

I = Intercept of sample

D.F = Dilution Factor

V = Volume

B = Slope of the standard curve

W = Weight of the sample

3.10 Determination of Antinutritional Factors

3.10.1 Determination of Tannin

One gram of each sample was weighed into a beaker. Each was soaked with solvent mixture (80 ml of acetone and 20 ml of glacial acetic acid) for 5 hours to extract tannin. The samples were filtered through a double layer filter paper to obtain the filtrates which were stored for further use. A standard solution of tannic acid was prepared ranging from 10ppm to 30ppm. The absorbances of the standard solution as well as that of the filtrates were read at 500nm on a spectronic 20, England spectrophotometer (AOAC, 1990).

3.10.2 Determination of Phytic Acid (Phytate)

Two gram of each sample was weighed into a 250ml conical flask. 100ml of 2% hydrochloric acid was used to soak each sample in a conical flask for 3 hours. This was filtered through a double layer of hardened filter paper Whattman No 3. 50ml of each filtrate was placed in 250ml beaker and 107ml of distilled water was added in each case. 10ml of 0.3% Ammonium Thiocyanate solution was added into each solution as indicator. This was titrated with standard Iron (III) chloride solution, which contained 0.00l95g iron per ml. The end point is slightly brownish yellow, which persisted for 5 minutes. The percentage phytic acid was calculated using the formula: -

$$\% \text{ Phytic acid} = \frac{X \times 1.19 \times 100}{0.00195}$$

Where X = Titre value

(AOAC, 1990).

3.10.3 Determination of Trypsin Inhibitor

0.2g of sample was weighed into a screw capped centrifuge tube. 10ml of 0.1M phosphate buffer was added and shaken vigorously. The contents were left at 25^0C for 1 hour on a UDY 60 shaker, England. The suspension obtained was centrifuged at 5000rpm for 5mins and filtered through Whatman No 42 filter paper. The volume of each was adjusted to 2ml with phosphate buffer. The test tubes were placed in a water bath, maintained at 37^0C. 6ml of 5% Tri chloro acetic acid (TCA) solution was added to one of the tubes to serve as a blank. 2ml of casein solution was added to all the tubes, which was previously kept at 37^0C. These were incubated for 20 minutes. The reaction was stopped after 20mins by adding 6ml of TCA solution to the experimental tubes and shaken. The reaction was left for 1 hour at room temperature after which it was filtered through Whatman No 42 filter paper. Absorbance of filtrate from sample and trypsin standard solutions was read at 380nm on a spectronic 20, England spectrophotometer. The trypsin inhibitor in mg/g sample was calculated using the formula:

$$\text{'Trypsin mg/g} = \frac{\text{A STD-A sample} \times \text{Dilution factor}}{19 \times \text{sample wt in g}} \times 1000$$

(AOAC, 1990)

3.10.4 Determination of Protease Inhibitor.

2% solution of egg albumin and 0.1% solution of Bromelain, both in pH 7 phosphate buffer was prepared. 5ml of the egg albumin substrate and 1ml of the Bromelain enzyme was incubated at 55^0C for 10 min. 5ml 10% TCA was added to stop the reaction. The precipitate was filtered off with Whatman No. 1 Filter paper and the absorbance of the filtrate was measured at 280nm on the Atomic Absorption Spectrophotometer, (AAS) labelled (Ai). The entire procedure was repeated but incubating with the enzyme and substrate mixture, i.e. 1ml of the extract of the material for protease inhibitor determination labelled (As). The absorbance of the filtrate was measured at 280nm. This was denoted Ai.

$$\% \text{ Protease Inhibitor} = \frac{\text{As-Ai} \times 100}{\text{As}}$$

Where As = Absorbance of sample

Ai = Absorbance of Blank/initial

(Cuatrecasas andAnfisen, 1991)

3.11 Determination of Total Oligosaccharide

The method of Black and Bagley (2007) was used for the determination of the total oligosaccharide content. One gram of ground defatted soybean meal was weighed into a 50ml polyethylene centrifuge tube (100x26mm). The sample was thoroughly mixed with 10ml ethanol-water (80:20 v/v) using a glass-stirring rod. The sample was heated in a water bath for 30mins with frequent stirring and centrifuged at 2000rpm for 5mins. The extraction process was repeated thrice, each time with the extract mixture in a 50ml beaker. The extract mixture was de-proteinized with 2ml of 10% lead acetate and centrifuged. The precipitate was washed with 3ml of the ethanol solution and re-centrifuged. The extracts were then hydrolysed with 0.1M HCl overnight. After this, the digest was diluted with acid to a suitable dilution factor. The raffinose- oligosaccharide was analysed by gas chromatography with flame ionization detector (GC-FID), Perkin Elmer Instrument, England. The readings obtained were then subjected to a standard raffinose -oligosaccharide curve for conversion to the equivalent sugar (Black and Bagley, 2007).

3.12 Genetic Characterization of Isolates

3.12.1 Extraction of Genomic DNA of LAB Isolates

DNA extraction from the LAB isolates was carried out using a modified GES (5M guanidine thiocyanate (Fisher scientific, England), 0.1 N EDTA (Sigma, England), and 0.5% N-lauroyl – sarcosine sodium salt (Sigma, England) (w/v) DNA extraction method (Pitcher *et al.*, 1989). Aliquots of 1.5ml of overnight cultures grown in appropriate broth were centrifuged (Biofuge, Heraeus, Germany) in Eppendorf tubes at 13,000g for 1min. Pellets obtained were washed in 1ml of ice cold lysis buffer (25mM Tris-Hcl (Sigma,England), 10mM EDTA, 50mM sucrose (BOH GPR 303997J), pH 8). The pellets were re-suspended in 100µl of lysis buffer in addition to $50mgml^{-1}$ lysozyme (Sigma, England.) and incubated at 37^0C for 30 min. Volumes of 0.5ml GES solution were added and mixed thoroughly. This was incubated at room temperature for 15min. The lysate was then cooled on ice for 2 min and 0.25ml of 7.5M ammonium acetate

(Fisher scientific, England). Cooled ice was also added, vortexed and incubated on ice for 10min. Aliquots (0.5ml) of 24:1 chloroform; isoamyalcohol (Sigma, England) were added, vortexed and centrifuged for 10min at 13,000g. Aliquots of 800ml of the upper phase were removed quantitively and placed in a clean Eppendorf tube. Cold isopropanol (Fisher scientific, England) was added and mixed for 1min.

This was then centrifuged at 13,000g for 5min and the supernatant removed from the pellet. The pellet was washed three times in 500µl of 70% ethanol and dried at 37^0C for 15min. Aliquots (50ul) of TE buffer were added and 5ul of the DNA were checked on 1% agarose (Biogene, Kimbolton, UK) gels in 200ml 1X TAE buffer and the DNA samples were then stored at – 20^0C for future use.

3.13 Polymerase Chain Reaction (PCR) amplification of 16S rDNA gene

The method of Bulut *et al*. (2005) was used. Amplification of 16S rDNA gene – ITS region, was performed by using the following primer pairs.

Forward (16S ITS For), 5′– AGAGTTTGATCCTGGCCTCAG-3′ and reverse (16S – ITS Rev), 5′ – CAAGGCATCCACCGT – 3′, 16S rDNA V3, forward 5′ – CCTAGGGGAGGCAGCAG – 3′ and 16S rDNA V3, reverse, 5′ – ARRACCGCGCTGCTGC-3′. The forward 5'-CCTACGGGAGGCAGCAG-3' and reverse, 5'-ATTACCGCGGCTGCTGG-3', primers used occupied positions 341–358 and 518–534 respectively of the V3 region in the 16S ribosomal DNA *of Escherichia coli*. The primers specify about 200 bp of the PCR products (as could be seen on the gel after electrophoresis)

The V3 primer pair was used for ease of sequencing of the gene, using the variable region 3 (V3), for the genetic identification of the isolates.

Each of the polymerase chain reactions (PCR) was performed in a 50µl reaction volume containing. 50µg genomic DNA as the template (equivalent to 1µl). 10µl 0.2mM deoxynucleoside triphospates, dNTPs (Promega UI20A – UI23A, Madison, WI, USA), 10 µl 2.5mM $MgCl_2$, 10pmol each (0.1µl volume) of the DNA primer in Ix PCR buffer (Promega, UK), and 10µl of 1.25 units Taq DNA polymerase (Promega, UK) and 18.9µl distilled water. Amplification conditions were as follows: an initial denaturation step of 5 min at 94^0C, 40 amplification cycles, each consisting of 1min denaturation at 94^0C, 1 min annealing at 42^0C, and 1min elongation at 72^0C. Reactions were terminated with a final extension step for 10min at 72^0C. PCR amplification was performed in a Thermocycler (Techne- Progene, Cambridge, UK).

3.14 Gel Electrophoresis of 16S rDNA PCR Products

Electrophoresis of the amplified 16s rDNA PCR products were performed on the Bio-Rad contour – clamped homogenous electric field (CHEF) DRII electrophoresis cell. This was done through 1.5% (w/v) agarose gel (Biogene, Germany) in 0.5 X TAE buffer at 84 volts for 1.5-2hrs. This was prepared by boiling 1.5 g of agarose powder in 100mls of 0.5X TAE buffer. A 100bp ladder (Promega, U.K) and 1Kb DNA ladder (Promega, U.K) were used as molecular size markers.

3.15 Sequencing and Analysis Of 16S rDNA Gene

3.15.1 Purification of PCR 16S rDNA Gene

75 µl of the PCR 16S rDNA amplified products (obtained above) were resolved in 1% agarose gels (agarose gel was prepared by boiling 1.0g of agarose powder in 100mls of 0.5 X TAE buffer) with the conditions earlier described above. PCR products were resolved by gel electrophoresis, using an agarose gel (1.5%; Biogene) that was stained with of 0.5µg/ml ethidium bromide, in 1xTAE buffer at 84 volts for 1.5 - 2 h.

The DNA bands were then visualised using a UV transilluminator (Amersham Pharmacia Biotech, UK) with 313 nm emission and pictures were taken using Fuji Film Imaging system FT1-500 (Amersham Pharmacia Biotech, UK).

The resulting bands in agarose gel were carefully excised with sterile scalpels and then purified the Wizard PCR preps DNA purification kit (Promega, USA).

The purification process involved the introduction of the excised bands into 1.5ml Eppendorf tubes which were suspended in a water bath that was maintained at 65^0C. 1ml PCR preps purification resin was added and the tubes were incubated for about 5 minutes until the agarose gel melted completely. The DNA purification resin was pipetted into the syringe barrel and the syringe plunger was used to slowly push the slurry into the attached minicolumn. The minicolumn was then washed with 2ml of 80% isopropanol, to remove contaminants from the DNA. The Eppendorf tubes, on which the 1.5ml minicolumns were mounted, were then centrifuged at 10,000g for 2 minutes at 4^0C, to remove remaining resin and isopropanol from the purified DNA in the columns.

The minicolumns were transferred into new Eppendorf tubes and 40µl TE buffer (pH 7.5) was added into each of them, and left to stand for 1 minute.

The minicolumns were centrifuged at 10,000g for 20secs at 4^0C to elute the DNA into the centrifuge tubes. The purified DNA was kept at 4^0C until used.

3.16 Qualitative and Quantitative Determination of Purified 16S rDNA Gene

This was done using software – NanoDrop, version 3.1.0, (Coleman technologies Inc, USA) according to the manufacturer's instructions.

3.17 Drying of the Purified 16S rDNA Genes

To a 50µl of the purified DNA, 0.1µl of sodium acetate buffer (3M, pH 5.0) and 2.0µl of 100% ethanol were added. This was then incubated at – 20^0C for 1hr. It was brought out and left to stand at room temperature for 5 mins, and then centrifuged at 13,000g at 4^0C for 45 mins. The liquid was removed, leaving only the DNA in the Eppendorf tubes. The DNA was dried in an incubator at 37^0C for 30 mins.

3.18 Sequencing of 16S rDNA Gene

The dry DNA samples (obtained using V3 primers) were sequenced using a computer analytical sequencer (MGW – Biotech, Germany) with the V3 and V5 primer Rev, acting as the basis according to manufacturer's instructions. The generated nucleotide sequences were subjected to analysis. Sequencing of the purified 16S rDNA DNA products was performed using the sequencing unit of the University of Nottingham; a 373 DNA sequence (Perkin-Elmer Applied Biosystems) was used with the Taq Dye Deoxy terminator cycle sequencing kit (Perkin-Elmer Applied Biosystems). The full identities of the isolates were then obtained by subjecting the nucleotide sequences to searches in the GenBank (http://www.ncbi.nlm.nih.gov/blast/) with the Blast search program.

PCR amplification conditions consisted of initial denaturation at 94^oC for 5 min, followed by 30 cycles of denaturation at 94^oC for 1 min, annealing at 55^oC for 30 s, extension at 72^oC for 45 s, and a final extension at 72^oC for 5 min. The amplification was performed in a Thermo cycler (Techne, Progene, Cambridge, UK). This was carried out in a 50 µl reaction volume containing 1.25 units of *Taq* DNA polymerase (ABgene, Thermo Fischer, UK), 2.5 mM magnesium chloride (Promega, UK), 0.2 mM ddNTPs (di-deoxy nucleotide tri phosphates; Promega), 0.1 µl of each reverse and forward primer, 5 µl PCR buffer and 5 µl of DNA template. Volume was made up with sterile deionised water.

3.19 Analysis of The 16S rDNA Gene Sequence

The generated sequences of the 16s rDNA genes were subjected to alignment in the databases at the BLAST, Basic Local Alignment and Search Tool, Website: http://www.ncbi.nih.gov/blast/. The isolates were then identified based on the result of the analysis.

3.20 Plasmid Analysis

3.20.1 Plasmid Isolation and Profiling:

Plasmid extraction was carried out using the method described by Brown (2000). Pure isolates were inoculated on MRS broth and incubated. The grown cells were harvested and suspended in 200µl of solution A (100 mM glucose-50 mM Tris hydrochloride (pH 8)-10 mM EDTA) containing 10 mg of lysozyme per ml and 10µg/ml mutanolysin and incubated for 30 min at 37°C in an incubator. 400µl of freshly prepared 1% sodium dodecyl sulfate in 0.2 N NaOH was added and the samples were mixed by inverting the Eppendorf tubes. 600µl of a 15% potassium acetate solution (pH 4.8) was added and the samples were mixed by vortexing. After incubating on ice for 5 minutes, the debris was removed by a 5-minute centrifugation in a centrifuge (model 5415R; Eppendorf). The supernatant was removed and extracted once with a phenol-chloroform mixture (1:1) and precipitated with an equal volume of isopropanol. The plasmid DNA was then dissolved in 100µl of TE buffer.

Electrophoresis of the DNA was carried out on a 0.8% agarose gel in a 0.5X concentration of Tris-Borate-EDTA (TBE) buffer. Agarose gel was prepared by boiling 0.8g of agarose powder in 100mls of 0.5X TBE buffer. After boiling, the solution was allowed to cool and 10µl of ethidium bromide was added to the cooled agarose solution. This was poured into a casting tray with a comb placed across its rim to form wells. The gel was allowed to set for 30 minutes and the comb was removed. 20µl of the plasmid DNA samples were then loaded into the wells after mixing with 2µl of bromophenol blue. A DNA molecular weight marker was also loaded into one of the wells. The gel was there after electrophoresed in a horizontal tank at a constant voltage of 60V for about 1 hour 30 minutes.

After electrophoresis, plasmid DNA bands were viewed by fluorescence of bound ethidium bromide under a short wave ultraviolet light trans-illuminator and the photographs were taken using a Samsung 12.2 Mega Pixel, Germany digital camera.

3.20.2 Plasmid Curing

The plasmids were cured by treatment with acridine orange according to the method of Brown (2000). Nutrient broth was prepared and supplemented with 0.1mg/ml acridine orange. 20µl of overnight culture of the bacteria was subcultured into 5 mls of the nutrient broth containing acridine orange. The samples were then incubated at 37°C for 72hours. After 72 hours incubation, the isolates were subcultured onto Mueller Hinton agar and plasmid extraction was repeated on some of the organisms to verify if the plasmid were successfully cured.

3.21.1.1 Preparation of the Cereal Blend

The three samples of the fermented gruels '*ogi'* were used for the formulation - white, yellow and brown (sorghum) *ogi*. It was prepared in the ratio 1:3 with the various soybeans prepared in different ways, namely:-raw, cooked and roasted. It was allowed to ferment with either the cured and uncured *L. plantarum* and the proximate analysis, antinutritional and residual oligosaccharides (and their profiles) and reducing sugars (and their profiles) were determined as described earlier. The food blend produced was also served as samples to nursing mothers and the organoleptic attributes of the various food blends such as taste, colour, aroma, texture, flavour, odour, shelf life, acceptability and appearance were assessed with questionnaires.

3.21.1.2 Packaging of the Weaning Food Blend

The method of Banigo and Muller (1972) was followed in the preparation of *ogi*. *Ogi* was made using clean fermented cereals. Fermentation was with cold water and this was left for 5 days. After this, it was milled and sieved. It was allowed to settle and stored in clean plastic container. The water was changed regularly to make it as fresh as desired and retain the desired taste, aroma and flavour.

Soybean was prepared by hand picking stones and dirt. It was roasted at 100^0C on a hot plate. It was broken into small pieces with a waring blender, the seed coat was removed and it was milled into powder and sieved through a 0.5 mm sieve. It was then stored in air tight plastic container and opened when ready to use. It was also sealed in nylon containers and stored at room temperature.

Ogi can be refrigerated for storage or left at room temperature and the fermenting water changed when it is ready to be consumed. This allows further fermentation to take place.

The blend or formulation was done when it was ready to be used. Three parts *ogi* sample was mixed with one part roasted soybeans sample. {This was prepared by mixing the blend together thoroughly one hour before cooking and served as desired to the infant}.

3.21.1.3 Oligosaccharide Determination in Soybean

3.21.2 Preparation of Inocula

The LAB isolates namely *L. plantarum* LV1, LV2 and LV3 that were used for the various tests were prepared by inoculating a colony from a 24hr- old culture of each *L. plantarum* streaked on a plate into a sterile 9ml MRS broth. This was incubated for 24hrs at 30^0C.

3.21.3 Standardization of Inocula

The LAB isolates that were used were standardized according to MacFarland standard using $BaCl_2$ and HCl at the right proportion. The culture supernant was also brought to the same optical density (OD) of 0.500 and a colony count of 3.1 $x10^3$ cfu/ml, using sterile MRS broth (Olutiola *et al.*, 1993).

3.21.4 Preparation of MRS Broth for Fermentation of Soybeans

MRS broth was prepared according to the method of De Mann Rogosa and Sharpe (1966). The media was sterilized in an autoclave for 15min at 121^0C and allowed to cool after which the test isolates were inoculated into the broth. Using a sterile pipette, the inoculum was introduced into the various soybeans samples as starters for fermentation.

3.21.5 Fermentation of Soybeans with Uncured and Cured Isolates of *L. plantarum*

Two batches each of one gram, two grams and thre grams each of the raw, cooked and roasted soybeans were weighed in triplicate into screw capped bottles. The first batch was used for the uncured isolates while the second batch was used for the cured isolates. 10 ml sterile distilled water was added to it for the sample to become a paste. 1ml each of the standardised inocula of cured and uncured *L.plantarum* were added separately to the two batches and allowed to ferment for five days. Samples were taken for reducing sugar and oligosaccharide content determination every 24 hours. A control was set up for the

samples with additional 1ml of sterile distilled water in the samples without the organisms.

3.22 Reducing Sugar Determination during fermentation of soybeans with *L. plantarum* isolate

During the fermentation of the soybean with both uncured and cured isolates respectively for 5 days, 1 ml each of the various samples was taken at 24 –hr intervals for the determination of the reducing sugar of the fermented samples. This was centrifuged at 3500 rpm for 15 mins after which the supernatant was used for reducing sugar determination according to the method of Bernfeld (1955) described in Appendix 3.3.

3.23 Determination of Residual Oligosaccharide Content in Fermented Soybean with *L.plantarum*

During the fermentation of the soybean with both uncured and cured isolates respectively for five days, two gram each of the various samples was taken at 24 –hr intervals for the determination of the residual oligosaccharide of the fermented samples according to the method of Black and Bagley, (2007).

3.24 Determination of Raffinose, Verbascose and Stachyose during fermentation of soybeans with uncured *L. plantarum* isolates

0.50 g of milled soybean seed (to pass a 0.5 mm sieve) and fermented samples were weighed accurately into glass test-tubes (18 x 150 mm). 5 ml of ethanol (95 % v/v) was added to each tube and incubated at 84-88^{0}C for 5 min to inactivate endogenous enzymes. The tube contents were quantitatively transferred to a 50 ml volumetric flask and the volume adjusted to the mark with 50 mM sodium acetate buffer (pH 4.5). The samples were allowed to extract over 15 min with occasional swirling. An aliquot (approx. 5 ml) of this slurry was transferred to a glass test tube (16 x 120 mm). 2 ml of chloroform was added and mixed vigorously on a vortex mixer for 15s. It was centrifuged at 1,500g for 10 min [this treatment removes most of the lipids from the aqueous phase into the chloroform (lower phase); insoluble plant material tends to concentrate between the phases]. The upper (aqueous) phase was analyed and diluted accordingly. The stepwise procedure stated in Appendix 3.4 was followed.

Methodology

The Oligossaccharide profile of the samples was determined by multiplying the absorbance difference of the blank and samples with the final volume of the mixture and molecular weight of the sample assayed.

The concentration of the oligossacharides:

c = V x MW x ΔA [g/L]

Where: V = final volume [mL]

MW = molecular weight of the substance assayed [g/mol]

= 6300 [l x mol-1 x cm -1]

v = sample volume [mL]

Raffinose:

c = 2.62 x 504.5 x Δ A raffinose [g/L]

= 6300 x 1 x 0.2

= 1.049 x ΔA raffinose [g/L]

Verbascose

c = 2.62 x 624.59 x Δ A verbascose [g/L]

= 6300 x 1 x 0.2

= 1.299 x ΔA verbascose [g/L]

Starchyose

c = 2.62 x 666.574 x Δ A stachyose [g/100g]

6300 x 1 x 0.2

= 1.386 x ΔA stachyose [g/L]

(Saini and Knights, (1984); Beutler, (1988).

3.25 Determination of Glucose, Fructose and Sucrose during fermentation of soybeans with uncured *L. plantarum* isolates

A) Preparation Of Fermented Soybeans Samples

0.50 g of milled (to pass a 0.5 mm sieve) and fermented seeds were weighed accurately into glass test-tubes. The samples were homogenized in distilled water and were filtered. It was extracted with hot water to remove the fat at a temperature above the melting point of the fat in a 100 ml volumetric flask. It was adjusted to 20°C and the volumetric flask was filled to the mark with water. It was stored in a refrigerator for 15-30 min and then filtered into different test tubes.

5 ml of ethanol (95 % v/v) `as added to each tube and incubated at 84-88°C for 5 min (this treatment inactivates endogenous enzymes). The tube contents were quantitatively transferred to a 50 ml volumetric flask and the volume adjusted to the mark with 50 mM sodium acetate buffer (pH 4.5). The samples were allowed to extract over 15 min with occasional swirling. An aliquot (approx. 5 ml) of this slurry was transferred to a glass test tube (16 x 120 mm). 2 ml of chloroform was added and mixed vigorously on a vortex mixer for 15s. It was centrifuged at 1,500g for 10 min [this treatment removes most of the lipids from the aqueous phase into the chloroform (lower phase); insoluble plant material tends to concentrate between the phases]. The upper (aqueous) phase was analyed and diluted according to the dilution table. The stepwise procedure stated in Appendix 3. 5 was followed.

B) Methodology

Calculation:

The reducing sugar profile of the samples was determined by multiplying the absorbance difference of the blank and samples with the final volume of the mixture and molecular weight of the sample assayed.

The concentration of the reducing sugars

Determination of sucrose:

The concentration of D-glucose, sucrose and D-fructose can be calculated as :

$c = V \times MW \times \Delta A$ [g/L]

***Where*:** V = final volume [mL]

MW = molecular weight of the substance assayed [g/mol]

= 6300 [l x mol-1 x cm-1]

v = sample volume [mL]

D-glucose:

$$c = \frac{2.42 \times 180.16 \times \Delta AD\text{-glucose}}{6300 \times 1 \times 0.1}$$ [g/L]

$= 0.6920 \times \Delta AD\text{-glucose}$ [g/L]

sucrose:

$$c = \frac{2.42 \times 342.3 \times \Delta AD\text{-sucrose}}{6300 \times 1 \times 0.1}$$ [g/L]

$= 1.315 \times \Delta A\text{sucrose}$ [g/L]

D-fructose:

c = 2.44 x 180.16 x ΔAD-fructose [g/L]

6300 x 1 x 0.1

= 0.6978 x ΔAD-fructose [g/L]

(Beutler, (1988); Outlaw and Mitchell, (1988).

3.26 Statistical Analysis Of Data

The various data obtained were analysed using Analysis of variance (ANOVA) and Duncan Multiple Range of variables and was performed using the general linear models of SAS 9.1 (SAS Institute Inc., Cary, NC, USA). Means of individual sugars in different physiological studies were compared and separated by SAS Waller grouping at $P = 0.05$.

CHAPTER FOUR

RESULTS

A total of 120 Lactic Acid bacteria were isolated from the various samples of *ogi* from three3 different varieties of the local white maize, yellow maize and sorghum, typed varieties ,Ex-Kano,Samsorg- 40 and Samsorg- 41 and three samples of *Ogi* obtained from commercial sources.

They were identified as *L. plantarum* (43); *L. brevis* (8), *L. acidophilus* (19), *L. casei* (7), *L. fermentum* (15), *L. pentosus* (9), *L. cellobiosus* (8), *L. jenseni* (6) and *L. reuterii* (5).

The appearance of the various colonies on modified MRS agar was circular, small and slightly raised. The margin was entire with shining surface and the colour was creamy. The edges were smooth and no pigment was produced. Also, the elevation was slightly raised. The Gram's reaction showed the cellular morphology. All the LAB isolates were Gram positive, some were short rods while others were long rods arranged either singly others or in pairs while some form chains and clusters.

The Biochemical tests showed that all were Catalase, Oxidase, Indole, Voges Proskaeur negative and exhibited a positive result for Methyl Red test. Some tested positive for Nitrate reduction, Hydrogen sulphide production and Citrate utilization. Growth in 4% NaCl and production of Ammonia from arginine also varies. The same trend was also observed in growth at a temperature of 15^0C and 45^0 C. All the isolates grew at pH 9.2, while some grew at pH 3.9, others grew in both pH ranges. All were able to hydrolyse starch but unable to hydrolyse casein and gelatine.

All the LAB isolates responded at varying degrees to the various sugar source used. Some fermented glucose with the production of gas but others did not. The various sugars used include glucose, lactose, maltose, fructose, sucrose, xylose, galactose, arabinose, rhamnnose, trehalose, raffinose, sucrose, dulcitol, mannitol and inositol. Their response to the sugars which was in varying degrees - positively, negatively, weakly or in a delayed manner was used to characterize the LAB isolates into species (Table 4.1) .

Table 4.2 shows the microbial load in colony forming unit per gram of the *ogi* samples produced from different maize and sorghum varieties. The microbial load of the local varieties LV1, LV2 and LV3 were 3.5 x 10^7 cfu/g,3.3 x 10^7 cfu/g and 3.2 x 10^7 cfu/g respectively.

This was followed by the isolates that were obtained from the commercial *ogi* samples 3.0 x 10^6 cfu/g, 3.1 x 10^6 cfu/g and 3.1 x 10^6 cfu/g representing C01, C02 and C03 respectively. The typed varieties TV1, TV2 and TV3 had the lowest microbial loads in cfu/g of 2.2 x 10^5, 2.1 x 10^5 and 2.1 x 10^5 respectively.

It was observed that the number of LAB isolates in the local varieties of maize and sorghum that were processed under controlled condition in the laboratory had the highest number of LAB isolates; this was followed by the commercial *ogi* sources that were obtained within Ibadan. The typed varieties have the lowest load. It was also observed that the typed varieties had the lowest microbial load.

Table 4.1: Biochemical and Physiological Characterization of the Isolates

Isolate code	Gram rxn	Cell . Mor	Catalase	Oxidase	Casein hydrolysis	Gel HND	M.R	V.P	H_2SP	Growth at 15^0C	45^0C	pH at 3.9	pH at 9.2	pH at 5	4% NaCl	CIT.UTI	Glucose	Xylose	Rhamnose	Triammonium citrate	Raffinose	Sucrose	Lactose	Maltose	Galactose	Fructose	Arabinnose	Mannose	Dulcitol	Mannitol	Inositol	Motility	Indole	NH_3Arg	Nitrate red	Probable identity
1	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	d	+	-	+	-	-	-	-	-	*Lactobacillus plantarum*
2	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	d	+	-	+	-	-	-	-	-	*Lactobacillus plantarum*
3	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	d	+	-	+	-	-	-	-	-	*Lactobacillus plantarum*
4	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	d	+	-	+	-	-	-	-	-	*Lactobacillus plantarum*
5	+	R	-	-	-	-	-	-	-	+	-	+	+	+	-	-	+G	D	-	-	W	D	W	+	W	+	+	-	-	W	-	-	-	+	+	*Lactobacillus brevis*
6	+	R	-	-	-	-	-	-	-	+	-	+	+	+	+	-	+	-	(d)	+	-	D	(-)	d	+	+	+	+	(+)	+	-	-	-	+	+	*Lactobacillus casei*
7	+	R	-	-	-	-	-	-	+	+	+	+	+	+	-	-	+	D	-	d	+	+	+	+	+	+	d	W	W	-	-	-	-	+	+	*Lactobacillus fermentum*
8	+	R	-	-	-	-	-	-	+	+	+	+	+	+	-	-	+	D	-	d	+	+	+	+	+	+	d	W	W	-	-	-	-	+	+	*Lactobacillus fermentum*
9	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	d	+	-	+	-	-	-	-	-	*Lactobacillus plantarum*
10	+	R	-	-	-	-	-	-	-	-	-	+	+	+	-	-	+	-	-	+	D	+	+	+	+	+	-	-	W	-	-	-	-	-	+	*Lactobacillus acidophilus*
11	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	d	+	-	+	-	-	-	-	-	*Lactobacillus plantarum*
12	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	d	+	-	+	-	-	-	-	-	*Lactobacillus plantarum*
13	+	R	-	-	-	-	-	-	-	+	-	-	+	+	-	-	+	+	-	-	+	+	+	+	+	+	+	(-)	(-)	+	d	-	-	+	+	*Lactobacillus pentosus*
14	+	R	-	-	-	-	-	-	-	+	-	-	+	+	-	-	+	+	-	-	+	+	+	+	+	+	+	(-)	(-)	+	d	-	-	+	+	*Lactobacillus pentosus*
15	+	R	-	-	-	-	-	-	+	-	-	+	+	+	-	+	+G	D	-	+	+	+	+	+	+	+	+	W	W	-	-	-	-	+	+	*Lactobacillus cellobiosus*
16	+	R	-	-	-	-	-	-	+	-	-	+	+	+	-	+	+G	D	-	+	+	+	+	+	+	+	+	W	W	-	-	-	-	+	+	*Lactobacillus cellobiosus*
17	+	R	-	-	-	-	-	-	-	+	-	+	+	+	-	-	+G	D	-	-	W	D	W	+	W	+	+	-	-	W	-	-	-	+	+	*Lactobacillus brevis*
18	+	R	-	-	-	-	-	-	-	-	-	+	+	+	-	-	+	-	-	+	D	+	+	+	+	+	-	-	W	-	-	-	-	-	+	*Lactobacillus acidophilus*
19	+	R	-	-	-	-	-	-	-	-	-	+	+	+	-	-	+	-	-	+	D	+	+	+	+	+	-	-	W	-	-	-	-	-	+	*Lactobacillus acidophilus*
20	+	R	-	-	-	-	-	-	-	-	-	+	+	+	-	-	+	-	-	+	D	+	+	+	+	+	-	-	W	-	-	-	-	-	+	*Lactobacillus acidophilus*
21	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	d	+	-	+	-	-	-	-	-	*Lactobacillus plantarum*

Table 4.1: Biochemical and Physiological Characterization of the Isolates

22	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	D	-	+	+	+	+	+	+	+	d	+	-	+	-	-	-	-	-	*Lactobacillus plantarum*
23	+	R	-	-	-	-	-	-	-	+	-	-	+	+	-	-	+	+	-	-	+	+	+	+	+	+	+	(-)	(-)	+	D	-	-	+	+	*Lactobacillus pentosus*
24	+	R	-	-	-	-	-	-	-	+	-	+	+	+	+	-	+	-	(d)	+	-	D	(-)	d	+	+	+	+	(+)	+	-	-	-	+	+	*Lactobacillus casei*
25	+	R	-	-	-	-	-	-	-	-	(+)	-	+	+	-	-	+	-	-	+	-	+	-	+	+	+	-	+	-	-	-	-	-	+	-	*Lactobacillus jensenii*
26	+	R	-	-	-	-	-	-	+	+	-	(+)	+	+	+	+	+	+	+	+	+	-	-	(+)	d	(+)	W	-	(+)	(+)	W	-	-	+	+	*Lactobacillus reuterii*
27	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	d	-	+	+	+	+	+	+	+	d	+	-	+	-	-	-	-	-	*Lactobacillus plantarum*
28	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	d	-	+	+	+	+	+	+	+	d	+	-	+	-	-	-	-	-	*Lactobacillus plantarum*
29	+	R	-	-	-	-	-	-	-	+	-	+	+	+	+	-	+	-	(d)	+	-	D	(-)	d	+	+	+	+	(+)	+	-	-	-	+	+	*Lactobacillus casei*
30	+	R	-	-	-	-	-	-	+	+	+	+	+	+	-	-	+	d	-	d	+	+	+	+	+	+	d	W	W	-	-	-	-	+	+	*Lactobacillus fermentum*
31	+	R	-	-	-	-	-	-	+	+	+	+	+	+	-	-	+	d	-	d	+	+	+	+	+	+	d	W	W	-	-	-	-	+	+	*Lactobacillus fermentum*
32	+	R	-	-	-	-	-	-	+	+	+	+	+	+	-	-	+	d	-	d	+	+	+	+	+	+	d	W	W	-	-	-	-	+	+	*Lactobacillus fermentum*
33	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	d	-	+	+	+	+	+	+	+	d	+	-	+	-	-	-	-	-	*Lactobacillus plantarum*
34	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	d	-	+	+	+	+	+	+	+	d	+	-	+	-	-	-	-	-	*Lactobacillus plantarum*
35	+	R	-	-	-	-	-	-	+	+	-	(+)	+	+	+	+	+	+	+	+	+	-	-	(+)	d	(+)	W	-	(+)	(+)	W	-	-	+	+	*Lactobacillus reuterii*
36	+	R	-	-	-	-	-	-	+	+	-	(+)	+	+	+	+	+	+	+	+	+	-	-	(+)	d	(+)	W	-	(+)	(+)	W	-	-	+	+	*Lactobacillus reuterii*
37	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	d	-	+	+	+	+	+	+	+	d	+	-	+	-	-	-	-	-	*Lactobacillus plantarum*
38	+	R	-	-	-	-	-	-	-	+	-	+	+	+	-	-	+G	d	-	-	W	D	W	+	W	+	+	-	-	W	-	-	-	+	+	*Lactobacillus brevis*
39	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	d	-	+	+	+	+	+	+	+	d	+	-	+	-	-	-	-	-	*Lactobacillus plantarum*
40	+	R	-	-	-	-	-	-	+	+	+	+	+	+	-	-	+	d	-	d	+	+	+	+	+	+	d	W	W	-	-	-	-	+	+	*Lactobacillus fermentum*
41	+	R	-	-	-	-	-	-	+	+	+	+	+	+	-	-	+	d	-	d	+	+	+	+	+	+	d	W	W	-	-	-	-	+	+	*Lactobacillus fermentum*
42	+	R	-	-	-	-	-	-	-	-	-	+	+	+	-	-	+	-	-	+	D	+	+	+	+	+	-	-	W	-	-	-	-	-	+	*Lactobacillus acidophilus*
43	+	R	-	-	-	-	-	-	-	-	-	+	+	+	-	-	+	-	-	+	D	+	+	+	+	+	-	-	W	-	-	-	-	-	+	*Lactobacillus acidophilus*
44	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	d	-	+	+	+	+	+	+	+	d	+	-	+	-	-	-	-	-	*Lactobacillus plantarum*
45	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	d	-	+	+	+	+	+	+	+	d	+	-	+	-	-	-	-	-	*Lactobacillus plantarum*
46	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	d	-	+	+	+	+	+	+	+	d	+	-	+	-	-	-	-	-	*Lactobacillus plantarum*
47	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	d	-	+	+	+	+	+	+	+	d	+	-	+	-	-	-	-	-	*Lactobacillus plantarum*

Table 4.1: Biochemical and Physiological Characterization of the Isolates

48	+	R	-	-	-	-	-	-	-	-	-	+	+	+	-	-	+	-	-	+	D	+	+	+	+	+	-	-	W	-	-	-	-	-	+	*Lactobacillus acidophilus*
49	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	d	-	+	+	+	+	+	+	+	d	+	-	+	-	-	-	-	-	*Lactobacillus plantarum*
50	+	R	-	-	-	-	-	-	-	-	(+)	-	+	+	-	-	+	-	-	+	-	+	-	+	+	+	-	+	-	-	-	-	-	+	-	*Lactobacillus jensenii*
51	+	R	-	-	-	-	-	-	+	+	+	+	+	+	-	-	+	d	-	d	+	+	+	+	+	+	d	W	W	-	-	-	-	+	+	*Lactobacillus fermentum*
52	+	R	-	-	-	-	-	-	-	+	-	+	+	+	-	-	+G	d	-	-	W	d	W	+	W	+	+	-	-	W	-	-	-	+	+	*Lactobacillus brevis*
53	+	R	-	-	-	-	-	-	-	+	-	+	+	+	+	-	+	-	(d)	+	-	d	(-)	d	+	+	+	+	(+)	+	-	-	-	+	+	*Lactobacillus casei*
54	+	R	-	-	-	-	-	-	-	-	-	+	+	+	-	-	+	-	-	+	D	+	+	+	+	+	-	-	W	-	-	-	-	-	+	*Lactobacillus acidophilus*
55	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	d	-	+	+	+	+	+	+	+	d	+	-	+	-	-	-	-	-	*Lactobacillus plantarum*
56	+	R	-	-	-	-	-	-	-	+	-	-	+	+	-	-	+	+	-	-	+	+	+	+	+	+	+	(-)	(-)	+	D	-	-	+	+	*Lactobacillus pentosus*
57	+	R	-	-	-	-	-	-	-	+	-	-	+	+	-	-	+	+	-	-	+	+	+	+	+	+	+	(-)	(-)	+	D	-	-	+	+	*Lactobacillus pentosus*
58	+	R	-	-	-	-	-	-	+	+	+	+	+	+	-	-	+	d	-	d	+	+	+	+	+	+	d	W	W	-	-	-	-	+	+	*Lactobacillus fermentum*
59	+	R	-	-	-	-	-	-	-	-	(+)	-	+	+	-	-	+	-	-	+	-	+	-	+	+	+	-	+	-	-	-	-	-	+	-	*Lactobacillus jensenii*
60	+	R	-	-	-	-	-	-	-	-	-	+	+	+	-	-	+	-	-	+	D	+	+	+	+	+	-	-	W	-	-	-	-	-	+	*Lactobacillus acidophilus*
61	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	d	-	+	+	+	+	+	+	+	d	+	-	+	-	-	-	-	-	*Lactobacillus plantarum*
62	+	R	-	-	-	-	-	-	-	-	-	+	+	+	-	-	+	-	-	+	D	+	+	+	+	+	-	-	W	-	-	-	-	-	+	*Lactobacillus acidophilus*
63	+	R	-	-	-	-	-	-	-	-	-	+	+	+	-	-	+	-	-	+	D	+	+	+	+	+	-	-	W	-	-	-	-	-	+	*Lactobacillus acidophilus*
64	+	R	-	-	-	-	-	-	-	+	-	+	+	+	-	-	+G	d	-	-	W	d	W	+	W	+	+	-	-	W	-	-	-	+	+	*Lactobacillus brevis*
65	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	d	-	+	+	+	+	+	+	+	d	+	-	+	-	-	-	-	-	*Lactobacillus plantarum*
66	+	R	-	-	-	-	-	-	-	+	-	+	+	+	+	-	+	-	(d)	+	-	d	(-)	d	+	+	+	+	(+)	+	-	-	-	+	+	*Lactobacillus casei*
67	+	R	-	-	-	-	-	-	-	-	(+)	-	+	+	-	-	+	-	-	+	-	+	-	+	+	+	-	+	-	-	-	-	-	+	-	*Lactobacillus jensenii*
68	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	d	-	+	+	+	+	+	+	+	d	+	-	+	-	-	-	-	-	*Lactobacillus plantarum*
69	+	R	-	-	-	-	-	-	-	+	-	+	+	+	-	-	+G	d	-	-	W	d	W	+	W	+	+	-	-	W	-	-	-	+	+	*Lactobacillus brevis*
70	+	R	-	-	-	-	-	-	+	+	-	(+)	+	+	+	+	+	+	+	+	+	-	-	(+)	d	(+)	W	-	(+)	(+)	W	-	-	+	+	*Lactobacillus reuterii*
71	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	d	-	+	+	+	+	+	+	+	d	+	-	+	-	-	-	-	-	*Lactobacillus plantarum*
72	+	R	-	-	-	-	-	-	-	+	-	+	+	+	+	-	+	-	(d)	+	-	d	(-)	d	+	+	+	+	(+)	+	-	-	-	+	+	*Lactobacillus casei*
73	+	R	-	-	-	-	-	-	+	+	+	+	+	+	-	-	+	d	-	d	+	+	+	+	+	+	d	W	W	-	-	-	-	+	+	*Lactobacillus fermentum*

Table 4.1: Biochemical and Physiological Characterization of the Isolates

74	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	d	-	+	+	+	+	+	+	+	d	+	-	+	-	-	-	-	-	*Lactobacillus plantarum*
75	+	R	-	-	-	-	-	-	-	-	-	+	+	+	-	-	+	-	-	+	d	+	+	+	+	+	-	-	W	-	-	-	-	-	+	*Lactobacillus acidophilus*
76	+	R	-	-	-	-	-	-	-	-	-	+	+	+	-	-	+	-	-	+	d	+	+	+	+	+	-	-	W	-	-	-	-	-	+	*Lactobacillus acidophilus*
77	+	R	-	-	-	-	-	-	-	+	-	+	+	+	-	-	+G	d	-	-	W	d	W	+	W	+	+	-	-	W	-	-	-	+	+	*Lactobacillus brevis*
78	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	d	-	+	+	+	+	+	+	+	d	+	-	+	-	-	-	-	-	*Lactobacillus plantarum*
79	+	R	-	-	-	-	-	-	+	-	-	+	+	+	-	+	+G	d	-	+	+	+	+	+	+	+	+	W	W	-	-	-	-	+	+	*Lactobacillus cellobiosus*
80	+	R	-	-	-	-	-	-	+	-	-	+	+	+	-	+	+G	d	-	+	+	+	+	+	+	+	+	W	W	-	-	-	-	+	+	*Lactobacillus cellobiosus*
81	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	d	-	+	+	+	+	+	+	+	d	+	-	+	-	-	-	-	-	*Lactobacillus plantarum*
82	+	R	-	-	-	-	-	-	-	-	(+)	-	+	+	-	-	+	-	-	+	-	+	-	+	+	+	-	+	-	-	-	-	-	+	-	*Lactobacillus jensenii*
83	+	R	-	-	-	-	-	-	-	-	(+)	-	+	+	-	-	+	-	-	+	-	+	-	+	+	+	-	+	-	-	-	-	-	+	-	*Lactobacillus jensenii*
84	+	R	-	-	-	-	-	-	-	-	-	+	+	+	-	-	+	-	-	+	d	+	+	+	+	+	-	-	W	-	-	-	-	-	+	*Lactobacillus acidophilus*
85	+	R	-	-	-	-	-	-	-	-	-	+	+	+	-	-	+	-	-	+	d	+	+	+	+	+	-	-	W	-	-	-	-	-	+	*Lactobacillus acidophilus*
86	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	d	-	+	+	+	+	+	+	+	d	+	-	+	-	-	-	-	-	*Lactobacillus plantarum*
87	+	R	-	-	-	-	-	-	+	+	+	+	+	+	-	-	+	d	-	d	+	+	+	+	+	+	d	W	W	-	-	-	-	+	+	*Lactobacillus fermentum*
88	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	d	-	+	+	+	+	+	+	+	d	+	-	+	-	-	-	-	-	*Lactobacillus plantarum*
89	+	R	-	-	-	-	-	-	-	+	-	-	+	+	-	-	+	+	-	-	+	+	+	+	+	+	+	(-)	(-)	+	D	-	-	+	+	*Lactobacillus pentosus*
90	+	R	-	-	-	-	-	-	-	+	-	-	+	+	-	-	+	+	-	-	+	+	+	+	+	+	+	(-)	(-)	+	D	-	-	+	+	*Lactobacillus pentosus*
91	+	R	-	-	-	-	-	-	-	-	-	+	+	+	-	-	+	-	-	+	d	+	+	+	+	+	-	-	W	-	-	-	-	-	+	*Lactobacillus acidophilus*
92	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	d	-	+	+	+	+	+	+	+	d	+	-	+	-	-	-	-	-	*Lactobacillus plantarum*
93	+	R	-	-	-	-	-	-	-	+	-	+	+	+	-	-	+G	d	-	-	W	d	W	+	W	+	+	-	-	W	-	-	-	+	+	*Lactobacillus brevis*
94	+	R	-	-	-	-	-	-	+	-	-	+	+	+	-	+	+G	d	-	+	+	+	+	+	+	+	+	W	W	-	-	-	-	+	+	*Lactobacillus cellobiosus*
95	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	d	-	+	+	+	+	+	+	+	d	+	-	+	-	-	-	-	-	*Lactobacillus plantarum*
96	+	R	-	-	-	-	-	-	+	-	-	+	+	+	-	+	+G	d	-	+	+	+	+	+	+	+	+	W	W	-	-	-	-	+	+	*Lactobacillus cellobiosus*
97	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	d	-	+	+	+	+	+	+	+	d	+	-	+	-	-	-	-	-	*Lactobacillus plantarum*
98	+	R	-	-	-	-	-	-	+	-	-	+	+	+	-	+	+G	d	-	+	+	+	+	+	+	+	+	W	W	-	-	-	-	+	+	*Lactobacillus cellobiosus*
[99]	+	R	-	-	-	-	-	-	-	+	-	+	+	+	+	-	+	-	(d)	+	-	d	(-)	d	+	+	+	+	(+)	+	-	-	-	+	+	*Lactobacillus casei*

Table 4.1: Biochemical and Physiological Characterization of the Isolates

100	+	R	-	-	-	-	-	-	+	+	+	+	+	+	-	-	+	d	-	d	+	+	+	+	+	+	d	W	W	-	-	-	-	+	+	*Lactobacillus fermentum*
102	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	d	-	+	+	+	+	+	+	+	d	+	-	+	-	-	-	-	-	*Lactobacillus plantarum*
103	+	R	-	-	-	-	-	-	-	-	-	+	+	+	-	-	+	-	-	+	d	+	+	+	+	+	-	-	W	-	-	-	-	-	+	*Lactobacillus acidophilus*
104	+	R	-	-	-	-	-	-	+	-	-	+	+	+	-	+	+G	d	-	+	+	+	+	+	+	+	+	W	W	-	-	-	-	+	+	*Lactobacillus cellobiosus*
105	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	d	-	+	+	+	+	+	+	+	d	+	-	+	-	-	-	-	-	*Lactobacillus plantarum*
106	+	R	-	-	-	-	-	-	+	+	+	+	+	+	-	-	+	d	-	d	+	+	+	+	+	+	d	W	W	-	-	-	-	+	+	*Lactobacillus fermentum*
107	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	d	-	+	+	+	+	+	+	+	d	+	-	+	-	-	-	-	-	*Lactobacillus plantarum*
108	+	R	-	-	-	-	-	-	+	+	+	+	+	+	-	-	+	d	-	d	+	+	+	+	+	+	d	W	W	-	-	-	-	+	+	*Lactobacillus fermentum*
109	+	R	-	-	-	-	-	-	-	+	-	-	+	+	-	-	+	+	-	-	+	+	+	+	+	+	+	(-)	(-)	+	D	-	-	+	+	*Lactobacillus pentosus*
110	+	R	-	-	-	-	-	-	-	+	-	-	+	+	-	-	+	+	-	-	+	+	+	+	+	+	+	(-)	(-)	+	D	-	-	+	+	*Lactobacillus pentosus*
111	+	R	-	-	-	-	-	-	-	-	-	+	+	+	-	-	+	-	-	+	d	+	+	+	+	+	-	-	W	-	-	-	-	-	+	*Lactobacillus acidophilus*
112	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	d	-	+	+	+	+	+	+	+	d	+	-	+	-	-	-	-	-	*Lactobacillus plantarum*
112	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	d	-	+	+	+	+	+	+	+	d	+	-	+	-	-	-	-	-	*Lactobacillus plantarum*
113	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	d	-	+	+	+	+	+	+	+	d	+	-	+	-	-	-	-	-	*Lactobacillus plantarum*
114	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	d	-	+	+	+	+	+	+	+	d	+	-	+	-	-	-	-	-	*Lactobacillus plantarum*
115	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	d	-	+	+	+	+	+	+	+	d	+	-	+	-	-	-	-	-	*Lactobacillus plantarum*
116	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	d	-	+	+	+	+	+	+	+	d	+	-	+	-	-	-	-	-	*Lactobacillus plantarum*
117	+	R	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	d	-	+	+	+	+	+	+	+	d	+	-	+	-	-	-	-	-	*Lactobacillus plantarum*
118	+	R	-	-	-	-	-	-	+	+	-	(+)	+	+	+	+	+	+	+	+	+	+	-	-	(+)	d	(+)	W	-	(+)	(+)	W	-	-	+	+	*Lactobacillus reuterii*
119	+	R	-	-	-	-	-	-	+	+	+	+	+	+	-	-	+	d	-	d	+	+	+	+	+	+	d	W	W	-	-	-	-	+	+	*Lactobacillus fermentum*
120	+	R	-	-	-	-	-	-	-	-	-	+	+	+	-	-	+	-	-	+	d	+	+	+	+	+	-	-	W	-	-	-	-	-	+	*Lactobacillus acidophilus*

Key: R= Rods
- = A negative reaction
d = A delayed reaction
(-) = A weakly negative reaction
+G = positive reaction and Gas production

+ = A positive reaction
W= A weak reaction
(+) = A weakly positive reaction
D= Extremely delayed reaction

Table 4.2: Microbial load (cfu/g) of the *Ogi* samples produced from different Maize and Sorghum Varieties

Sample code	Source of *ogi* sample	Microbial load
LV1	Local variety 1 (Wo)	3.5×10^7
LV2	Local variety 2 (Yo)	3.3×10^7
LV3	Local variety 3 (So)	3.2×10^7
TVI	Types variety 1 (Ex Kano)	2.2×10^5
TV2	Typed variety 2 (Samsorg 40)	2.1×10^5
TV3	Typed variety 3 (Samsorg 41)	2.1×10^5
C01	Commercial *Ogi* (W2)	3.2×10^6
C02	Commercial *Ogi* (Y2)	3.1×10^6
C02	Commercial *Ogi* (S2)	3.1 x 106

Table 4.3 shows the frequency of occurrence of the LAB isolates from *ogi* samples produced from different maize and sorghum varieties. *L. plantarum* had the highest percentage (35.8%) ,followed by *L. acidophilus* (15.8%) and *L. fermentum* (12.5%).The least occurring were *L. jensenii* with 5% and *L. renterii* (4.2%). Analysis of variance shows significance difference (p=0.05) in all the isolates used.

Table 4.4 shows the fermentation of all the isolates in a complex medium in which glucose was substituted with equal amounts (w/v) with lactose and raffinose. All the isolates were subjected to fermentation in the complex medium. This was a form of screening to choose the isolates that will be able to ferment the complex medium. The result showed that *L. plantarum* has the highest optical density in glucose, lactose and raffinose with value of 2.9, 2.2 and 1.9 respectively.

This was followed by *L. acidophilus* with optical density of 2.5, 2.2 and 1.7 in glucose lactose and raffinose respectively. The organisms that were able to ferment the sugar least were *L. reuterii* and *L. brevis.* Analysis of variance of isolates by sugars used showed a significant difference (p=0.05) with glucose being the highest, this was followed by, lactose and the least was raffinose (2.1, 1.5, and 1.3) respectively. All the organisms fermented the sugars in the same order. Glucose (2.9, 2.5) and (2.1, 2.0) lactose (2.2, 2.2) and (1.5, 1.1) while raffinose was the least (1.9, 1.7) and (1.3, 1.0) by *L. acidophilus, L. reuterii* and *L.brevis* respectively.

This result shows that the organisms were able to ferment glucose better than lactose and raffinose. Raffinose was the least in the optical densities obtained from the isolates. Analysis of variance of sugars by isolates shows a significance difference (p=0.05) for all the isolates used.

Table 4.5 shows the production of the antimicrobials lactic acid, hydrogen peroxide and diacetyl (mg/L) by the isolates from different gruels. All the isolates were subjected to this test but the nine that produced these antimicrobial agents in abundance were chosen for further work. These are *L. plantarum* TV1, *L. plantarum* TV2, *L. plantarum* TV3, *L. plantarum* LV1 and *L. plantarum* LV3, *L. plantarum* C01, *L. plantarum* C02 and *L. plantarum* C03. All the other organisms did not produce the antimicrobials as much these and this was why all the nine *L. plantarum* were chosen for further work.

Table 4.6 shows the percentage composition of samples and the number of organisms isolated from them. There were more isolates from the local varieties (60 in all) than from the commercial varieties (40). Only 20 organisms were isolated from the typed varieties.

The local varieties had 50% of the total isolates, 33.3% were from the commercial varieties while only 16.7% were isolated from the typed varieties.

Table 4.3: Frequency of Occurrence (%) of the LAB Isolates in Ogi Samples produce from different Maize and Sorghum Varieties

Isolates	No of Isolates	% Occurrence
L. plantarum	43	35.8%
L. brevis	08	6.7%
L. acidophilus	19	15.8%
L.casei	07	5.8%
L. fermentum	15	12.5%
L.pentosus	09	7.5%
L.cellobiosus	08	6.7%
L. jensenii	06	5.0%
L.reuterii	05	4.2%
Total	120	100

Table 4.4: Residual sugar quality/ fermentation of a composition of complex medium by the different LAB isolates

	Sugars		
Isolate	Glucose (mg/ml)	Lactose	Raffinose
L. fermentum	2.431	1.864	1.621
L. acidophilus	2.526	2.202	1.742
L. plantarum	2.852	2.212	1.862
L.pentosus	2.248	1.841	1.524
L. cellobiosus	2.228	1.742	1.511
L.jensenii	2.212	1.628	1.402
L. reuterii	2.104	1.541	1.321
L. casei	2.086	1.326	1.108
L. brevis	2.028	1.141	1.021

*Each value is a mean of triplicate determinations with Standard Error

Table 4.5: Time Course of Production of Lactic Acid, Hydrogen Peroxide and Diacetyl (g/L) by LAB Isolates from Different Cereal Gruels

	Lactic Acid			Hydrogen Peroxide			Diacetyl		
	Time Interval								
Code	24hrs	48hrs	72hrs	24hrs	48hrs	72hrs	24hrs	48hrs	72hrs
TV1	*1.32±0.003	1.50±0.005	1.57±0.002	0.0012±0.015	0.0010±0.002	0.0010±0.003	0.57±0.002	0.52±0.010	0.47±0.003
TV2	1.30± 0.025	1.42±0.015	1.48±0.033	0.0012±0.002	0.0013±0.010	0.0010±0.003	0.54± 0.020	0.48±0.033	0.42±0.025
TV3	1.29±0.010	1.32±0.005	1.36±0.025	0.0017±0.015	0.0015±0.010	0.0012±0.015	0.50 ±0.002	0.44±0.025	0.40±0.002
LV1	1.45±0.002	1.56±0.025	1.56±0.010	0.0030±0.020	0.0024±0.025	0.0020±0.025	0.87±0.005	0.80±0.002	0.40±0.020
LV2	1.41±0.003	1.47±0.003	1.52±0.010	0.0026±0.002	0.0020±0.033	0.0018±0.010	0.80±0.005	0.74±0.015	0.77±0.015
LV3	1.45±0.010	1.45±0.025	1.50±0.003	0.0024±0.010	0.0018±0.013	0.0014±0.010	0.78± 0.015	0.72±0.010	0.62±0.033
CO1	1.25±0.033	1.30±0.010	1.35±0.002	0.0018±0.020	0.0014±0.025	0.0011±0.010	0.54±0.010	0.50±0.003	0.46±0.033
C02	1.28±0.010	1.35±0.003	1.40±0.010	0.0014±0.005	0.0011±0.015	0.0010±0.033	0.53±0.020	0.47±0.015	0.43±0.003
C03	1.30±0.015	1.33±0.003	1.39±0.015	0.0014±0.005	0.0011±0.002	0.0010±0.020	0.46±0.002	0.43±0.033	0.40±0.025

*Each value is a mean of triplicate determinations with Standard Error

Table 4.6: Frequency of occurrence (%) of isolates relative to type of samples

Local Varieties	**No of Isolates**	**Total**	**Percentage**
Local Sorghum	40		
Local White maize	10		
Local Yellow Maize	10	60	50%
Commercially–Hawked *Ogi* Samples			
Commercial Sorghum *Ogi*	25		
Commercial White *Ogi*	7		
Commercial Yellow *Ogi*	8	40	33.3%
Typed Varieties			
Ex – Kano	10		
Samsorg 40	5		
Samsorg – 41	5	20	16.7%

Table 4.7 shows the extent of production of α-galactosidase enzyme by the different isolates. It was observed that *L. plantarum* from the various sources and varieties produced the enzyme in abundance compared to the other organisms. Isolates 9, 46, 55, 65, 86, 95, 101, 111 and 116 were selected for further studies. All the isolates produced the enzyme α-galactosidase above 1.0unit/ml. The values obtained were 1.8, 1. 8, 1.8 unit/ml for the three isolates from the local varieties while the ones from commercial sources gave 1.2, 1.2 and 1.2 unit/ml respectively. Isolates from the typed varieties produced 1.1, 1.1 and 1.1 unit per ml. These isolates were chosen for further work because of their ability to produce α-galactosidase in abundance. They were also chosen from the varieties/sources used for isolates so that the organisms from various sources will be represented. The organisms were later labelled as

L. plantarum LV1, LV2, LV3, C01, C02 and C03, TV1, TV2 and TV3.

Analysis of variance of these isolates used based on varieties shows a significance difference (p=0.05) in all the isolates used. There was a significance difference (p=0.05) between the local, commercial and typed varieties respectively. The production of α-galactosidase enzyme also shows a significance difference (p=0.05) in the isolates.

The production of alpha galactosidase from the organisms varies a lot; those that produced this in abundance were chosen for further work from all the sources.

Table 4.8 shows the production of α-galactosidase (unit/ml) enzyme by the selected *L. plantarum* cured and uncured isolates before and after curing of the isolates. LV1, LV2 and LV3 produced α-galactosidase in abundance before curing (1.8 unit/ml) of the isolates. After curing, the cured isolates produced a reduced amount of α-galactosidase (1.1. unit/ml).

Table 4.7: Extent of α- Galactosidase enzyme (Unit/ml) production by different LAB Isolates in complex medium

S/N	Isolate Code	Name	Extent of α-Galactosidase Produced (units/ml)
1.	LS1:	*L. plantarum*	0.962
2.	LS2:	*L. plantarum*	0.942
3.	LS3:	*L. plantarum*	0.963
4.	LS4:	*L. plantarum*	0.947
5.	LS5:	*L. brevis*	0.943
6.	LS6:	*L. casei*	0.921
7.	LS7:	*L. fermentum*	0.963
8.	LS8:	*L. fermentum*	0.814
9.	LS9:	*L. plantarum*	1.818
10.	LS10:	*L. acidophilus*	0.902
11.	LS11:	*L. plantarum*	0.940
12.	LS12:	*L. plantarum*	0.921
13.	LS13:	*L. pentosus*	0.872
14.	LS14:	*L. pentosus*	0.847
15.	LS15:	*L. cellobiosus*	0.867
16.	LS16:	*L. cellobiosus*	0.842
17.	LS1:7	*L. brevis*	0.810
18.	LS18:	*L. acidophilus*	0.810
19.	LS19:	*L. acidophilus*	0.934
20.	LS20:	*L. acidophilus*	0.926
21.	LS21:	*L. plantarum*	0.938
22.	LS22:	*L. plantarum*	0.940
23.	LS23:	*L. pentosus*	0.821
24.	LS24:	*L. casei*	0.830
25.	LS25:	*L. jensenii*	0.812
26.	LS26:	*L. reuterii*	0.810
27.	LS27:	*L. plantarum*	0.872
28.	LS28:	*L. plantarum*	0.890
29.	LS29:	*L. casei*	0.862

Table 4.7: Extent of α- Galactosidase enzyme (Unit/ml) production by different LAB

30.	LS30:	*L. fermentum*	0.914
31.	LS31:	*L. fermentum*	0.812
32.	LS32:	*L. fermentum*	0.814
33.	LS33:	*L. plantarum*	0.900
34.	LS34:	*L. plantarum*	0.920
35.	LS35:	*L. reuterii*	0.931
36.	LS36:	*L. reuterii*	0.914
37.	LS37:	*L. plantarum*	0.912
38.	LS38:	*L. brevis*	0.862
39.	LS39:	*L. plantarum*	0.879
40.	LS40:	*L. fermentum*	0.865
41.	LW1:	*L. fermentum*	0.812
42	LW2:	*L. acidophilus*	0.874
43.	LW3:	*L. acidophilus*	0.860
44.	LW4:	*L. plantarum*	0.914
45.	LW5:	*L. plantarum*	0.900
46.	LW6:	*L. plantarum*	1.820
47.	LW7:	*L. plantarum*	0.820
48.	LW8:	*L. acidophilus*	0.840
49.	LW9:	*L. plantarum*	0.912
50.	LW10:	*L. jensenii*	0.900
51.	LY1:	*L. fermentum*	0.926
52.	LY2:	*L. brevis*	0.845
53.	LY3:	*L. casei*	0.862
54.	LY4:	*L. acidophilus*	0.874
55.	LY5:	*L. plantarum*	1.805
56.	LY6:	*L. pentosus*	0.812
57.	LY7:	*L. pentosus*	0.816
58.	LY8:	*L. fermentum*	0.874
59.	LY9:	*L. jensenii*	0.810
60.	LY10:	*L. acidophilus*	0.883
61.	CS1:	*L. plantarum*	0.864
62.	CS2:	*L. acidophilus*	0.854

Table 4.7: Extent of α- Galactosidase enzyme (Unit/ml) production by different LAB

63.	CS3:	*L. acidophilus*	0.900
64.	CS4:	*L. brevis*	0.912
65.	CS5:	*L. plantarum*	1.217
66.	CS6:	*L. casei*	0.826
67.	CS7:	*L. jensenii*	0.814
68.	CS8:	*L. plantarum*	0.824
69.	CS9:	*L. brevis*	0.845
70.	CS10:	*L. reuterii*	0.820
71.	CS11:	*L. plantarum*	0.892
72.	CS12:	*L. casei*	0.840
73.	CS13:	*L. fermentum*	0.850
74.	CS14:	*L. plantarum*	0.872
75.	CS15:	*L. acidophilus*	0.900
76.	CS16:	*L. acidophilus*	0.912
77.	CS17:	*L. brevis*	0.884
78.	CS18:	*L. plantarum*	0.894
79.	CS19:	*L. cellobiosus*	0.824
80.	CS20:	*L. cellobiosus*	0.820
81.	CS21:	*L. plantarum*	0.092
82.	CS22:	*L. jensenii*	0.812
83.	CS23:	*L. jensenii*	0.814
84.	CS24:	*L. acidophilus*	0.864
85.	CS25:	*L. acidophilus*	0.862
86.	CW1:	*L. plantarum*	1.212
87.	CW2:	*L. fermentum*	0.847
88.	CW3:	*L. plantarum*	0.910
89.	CW4	*L. pentosus*	0.847
90.	CW5	*L. pentosus*	0.843
91.	CW6	*L. acidophilus*	0.862
92.	CW7	*L. plantarum*	0.842
93.	CY1	*L. brevis*	0.814
94.	CY2	*L. cellobiosus*	0.827
95.	CY3	*L. plantarum*	1.202

Table 4.7: Extent of α- Galactosidase enzyme (Unit/ml) production by different LAB

96.	CY4	*L. cellobiosus*	0.847
97.	CY5	*L. plantarum*	0.894
98.	CY6	*L. cellosbiosus*	0.824
99.	CY7	*L. casei*	0.814
100.	CY8	*L. fermentum*	0.878
101.	EXK-1	*Tv1L. plantarum*	1.114
102.	EXK-2	*L. acidophilus*	0.834
103.	EXK-3	*L. cellobiosus*	0.822
104.	EXK-4	*L. plantarum*	0.869
105.	EXK-5	*L. fermentum*	0.846
106.	EXK-6	*L. plantarum*	0.910
107.	EXK-7	*L. fermentum*	0.814
108.	EXK-8	*L. pentosus*	0.826
109.	EXK-9	*L. pentosus*	0.862
110.	EXK-10	*L. acidophilus*	0.874
111.	SAM 40-1	*TV2.L. plantarum*	1.102
112.	SAM 40-2	*L. plantarum*	0.854
113.	SAM 40-3	*L. plantarum*	0.821
114.	SAM 40-4	*L. plantarum*	0.846
115.	SAM 40-5	*L. plantarum*	0.852
116.	SAM 41-1	*Tv3 L. plantarum*	1.108
117.	SAM 41-2	*L. plantarum*	0.820
118.	SAM 41-3	*L. reuterii*	0.840
119.	SAM 41-4	*L. fermentum*	0.826
120.	SAM 41-5	*L. acidophilus*	0.822

*Each value is a mean of triplicate determinations ± Standard Error

Table 4.8: Production of α- Galactosidase Enzyme (Unit/ml) by selected cured and uncured *L.plantarum* Isolates

Isolate code	Conc. (units/ml)	
	Uncured	**Cured**
L.plantarum TV 1	*1.114±0.020	0.841±0.025
L.plantarum TV 2	1.102±0.003	0.852±0.003
L.plantarum TV 3	1.108±0.025	0.848±0.020
L.plantarum Lv1	1.818±0.002	1.006±0.020
L.plantarum Lv2	1.820±0.025	1.112±0.010
L.plantarum Lv3	1.805±0.010	1.100±0.025
L.plantarum Co1	1.217±0.020	0.922±0.025
L.plantarum Co2	1.212±0.005	0.918±0.003
L.plantarum Co3	1.202±0.003	0.912±0.005

*Each value is a mean of duplicate determinations with Standard Error

Table 4.9a shows the effect of different carbon sources on the selected *L. plantarum* on α-galactosidase production (unit/ml) using uncured isolates. LV1, LV2 and LV3 produced the highest in glucose (1.9, 1.9 and 2.0 unit/ml) respectively. This was followed by lactose (*1.3, 1.3, 1.3 unit/ml) respectively. In glucose, α-galactosidase production was (1.1, 1.0 and 1.0) unit/ml respectively. The least was observed in raffinose (0.8, 0.8 and 0.8) respectively.

Table 4.9b shows effect of different carbon sources on α-galactosidase (unit/ml) by *L. plantarum* using the cured isolates. There was a significant reduction (p=0.05) in the production of α-galactosidase when the cured isolate was used. Even though LV1, LV2 and LV3 were the highest in glucose when compared to uncured isolate, there was a reduction from 1.9 unit/ml to 1.4 unit/ml. In lactose, there was a reduction from 1.3 unit/ml to 0.9 unit/ml. In sucrose, there was a reduction from 1.1 to 0.8 unit/ml. The least was observed in raffinose from 0.8 unit/ml to 0.6 unit/ml.

Table 4.10 shows the production of amylase enzyme (unit/ml) by *L. plantarum* using the cured and uncured isolates LV1, LV2 and LV3 were the highest with 1.2, 1.1 and 1.1 respectively. The cured isolated produced 0.7, 0.7 and 0.7 unit/ml respectively. There was a significant reduction (p=0.05) in the production of amylase enzyme by the isolates

Table 4.9a: Effect of Different Carbon Sources on α-Galactosidase Production (Unit/ml) by uncured *L. plantarum* isolates

Isolate code	Carbon Source/Alpha-galactosidase concentration (Units/mL)			
	Glucose	**Lactose**	**Sucrose**	**Raffinose**
L.plantarum TV 1	*1.626±0.002	1.104±0.002	0.9481± 0.002	0.712±0.002
L.plantarum TV 2	1.644±0.003	1.100±0.005	0.960±0.010	0.711±0.010
L.plantarum TV 3	1.661±0.005	1.114±0.005	0.942±0.005	0.702±0.002
L.plantarum LV1	1.926±0.003	1.310±0.005	1.100±0.002	0.852±0.010
L.plantarum LV2	1.944±0.003	1.329± 0.050	1.006±0.010	0.843±0.002
L.plantarum LV3	1.951±0.005	1.285±0.005	1.000±0.005	0.832±0.004
L.plantarum CO1	1.576±0.005	1.046±0.005	0.912±0.005	0.700±0.005
L.plantarum CO2	1.543±0.002	1.071±0.050	0.924±0.005	0.704±0.005
L.plantarum CO3	1.524±0.005	0.989±0.003	0.920±0.005	0.701±0.005

*Each value is a mean of duplicate determinations with Standard Error

Table 4.9b: Effect of Different Carbon Sources on Alpha-Galactosidase (Units/mL) Production by cured *L. plantarum* isolates

Isolate code	Carbon Source/Alpha-galactosidase concentration (Units/mL)			
	Glucose	**Lactose**	**Sucrose**	**Raffinose**
L.plantarum C01	*1.013±0.005	0.874±0.005	0.712± 0.005	0.523±0.002
L.plantarum C02	1.000±0.025	0.865±0.002	0.707±0.013	0.5441±0.005
L.plantarum C03	1.003±0.003	0.867±0.010	0.720±0.005	0.571±0.010
L.plantarum LV1	1.324±0.025	0.997±0.005	0.819±0.002	0.617.±0.010
L.plantarum LV2	1.410±0.025	0.986±0.013	0.826±0.002	0.687±0.003
L.plantarum LV3	1.317±0.013	0.965±0.005	0.813±0.005	0.645±0.003
L.plantarum TV1	1.062±0.002	0.767±0.002	0.700±0.005	0.512±0.025
L.plantarum TV2	1.041±0.002	0.812±0.050	0.712±0.004	0.523±0.025
L.plantarum TV3	1.021±0.015	0.808±0.003	0.707±0.002	0.520±0.020

*Each value is a mean of duplicate determinations with Standard Error

Table 4.10: Production of Amylase Enzyme (Unit/ml) by selected cured and uncured *L.plantarum* isolates

Isolate code	Form of Isolate/Amylase Concentration (Unit/mL)	
	Uncured	**Cured**
L.plantarum TV1	*0.816±0.005	0.542±0.003
L.plantarum TV2	0.804±0.005	0.535±0.004
L.plantarum TV 3	0.810±0.002	0.542±0.025
L.plantarum LV1	1.214±0.003	0.714±0.005
L.plantarum LV2	1.116±0.002	0.721±0.005
L.plantarum LV3	1.106±0.005	0.720±0.025
L.plantarum Co1	0.826±0.005	0.620±0.003
L.plantarum Co2	0.814±0.025	0.620±0.002
L.plantarum Co3	0.802±0.002	0.623±0.002

*Each value is a mean of duplicate determinations with Standard Error

Table 4.11 shows the production of the mellibiase enzyme (unit/ml) by the cured and uncured *L. plantarum* isolates. It could be observed that LV1, LV2 and LV3 produced the highest (0.9, 0.8 and 0.8) respectively. The cured isolated produce (0.7, 0.7 and 0.7 unit/ml) respectively. Curing affected the production of mellibiase enzyme by the isolates significantly.

Table 4.12 shows the production of invertase enzyme (unit/ml) by the selected *L.plantarum* isolates using the cured and uncured isolates. LV1, LV2 and LV3 produced the highest (0.7, 0.7 and 0.7 unit/ml) while curing reduced the amount of the enzyme produced to 0.4, 0.4 and 0.4 unit/ml respectively. Curing significantly affected the production of invertase enzyme by the isolates.

From the tables 4.7-4.12, it could be observed that the production of all the enzymes by the selected *L.plantarum* isolates followed the same trend, Organisms isolated from the Local varieties produced all the enzymes in abundance, this was followed by the organisms isolated from commercial sources while the organisms isolated from the typed varieties produced the least. Also, the production of the enzymes was affected by plasmid curing. After curing, the production of the enzymes by the isolates reduced drastically.

Table 4.13 shows the production of crude protein (mg/ml) by the selected *L. plantarum* isolates using the uncured and cured isolates. LV1, LV2 and LV3 produced the highest (2.4, 2.2 and 2.2 mg/ml) respectively. There was a drastic reduction in the protein content production of the isolates when the cured isolates were used (1.3, 1.2 and 1.3) respectively. Curing significantly ($p=0.05$) affected the production of protein by the isolates.

The result obtained in Table 4.13 showed that the production of protein was also affected by plasmid curing because there was significant difference ($p=0.05$) in the protein content produced before curing and after curing by all the isolates. Furthermore, organisms isolated from the local sources produced more protein than those isolated from commercial sources while the ones isolated from typed varieties produced the least before and after curing.

Table 4.11: Production of Mellibiase Enzyme (Unit/ml) by the selected cured and uncured *L. plantarum* isolates

Isolate code	Conc. (unit/mL)	
	Uncured	**Cured**
L.plantarum Tv1	*0.762±0.003	0.542±0.003
L.plantarum Tv2	0.710±0.004	0.535±0.004
L.plantarum TV 3	0 .742±0.025	0.542±0.025
L.plantarum Lv1	0.864±0.005	0.714±0.005
L.plantarum Lv2	0.842±0.005	0.721±0.005
L.plantarum Lv3	0.840±0.025	0.720±0.025
L.plantarum Co1	0.816±0.003	0.620±0.003
L.plantarum Co 2	0.810±0.002	0.620±0.002
L.plantarum Co3	0.802±0.002	0.623±0.002

*Each value is a mean of duplicate determinations ± Standard Error

Table 4.12: Production of Invertase Enzyme (Unit/mL) by the selected cured and uncured *L. plantarum*

	Conc. (unit/ml)	
Isolate code	**Uncured**	**Cured**
L. plantarum Tv1	*0.470±0.002	0.320±0.002
L. plantarum Tv2	0.468±0.002	0.314±0.002
L. plantarum Tv3	0.467±0.002	0.356±0.002
L. plantarum Lv1	0.672±0.005	0.423±0.005
L. plantarum Lv2	0.660±0.005	0.420±0.005
L. plantarum Lv3	0.665±0.005	0.414±0.005
L. plantarum C01	0.480±0.003	0.334±0.003
L. plantarum C02	0.476±0.025	0.321±0.025
L. plantarum C03	0.478±0.003	0.318±0.003

*Each value is a mean of duplicate determinations ± Standard Error

Table 4.13: Production of Crude Protein (mg/ml) By the Selected Cured and Uncured *L.plantarum*

	Protein Conc. (mg/ml)	
Isolate code	**Uncured**	**Cured**
L. plantarum Tv1	*1.728±0.003	0.924±0.003
L. plantarum Tv2	1.710±0.005	0.918±0.005
L. plantarum Tv3	1.702±0.005	0.912±0.005
L. plantarum Lv1	2.442±0.002	1.342±0.002
L. plantarum Lv2	2.216±0.005	1.246±0.005
L. plantarum Lv3	2.210±0.025	1.256±0.025
L. plantarum C01	1.846±0.003	0.958±0.003
L. plantarum C02	1.824±0.025	0.936±0.025
L. plantarum C03	1.820±0.005	0.940±0.005

*Each value is a mean of duplicate determinations with Standard Error

Table 4.14 shows the production of reducing sugars in mg/ml by the *L. plantarum* isolate using cured and uncured samples. The uncured samples produced the highest LV1, LV2 and LV3: 1.2, 1.2 and 1.2 mg/ml respectively curing reduced the production of reducing sugar by the isolates to 0.9, 0.8 and 0.8 respectively. There was significant difference (p=0.05) in the amount of reducing sugar produced by the cured and uncured isolates.

From the result obtained, reducing sugar production was also affected by plasmid curing as there was significant difference (p=0.05) in the reducing sugar content produced before curing and after curing by all the isolates. Furthermore, organisms isolated from the local sources produced more reducing sugars than those isolated from commercial sources while the ones isolated from typed varieties produced the least before and after curing.

Table 4.15 shows the production of Beta- galactosidase enzyme by the isolates using cure and uncured LV1, LV2 and LV3 produced the highest 0.9, 0.9 and 0.9 respectively unit/ml. While there was a reduction when the cured isolates were used to 0.6, 0.6 and 0.6 respectively. Curing affected Bata-galactosidase production by the enzymes significantly (p=0.05).

From the result below, beta-galactosidase enzyme production was also affected by plasmid curing because there was significant difference (p=0.05) in the quantity of beta galactosidase enzyme produced before curing and after curing by all the isolates. Furthermore, organisms isolated from the local sources produced more reducing sugars than those isolated from commercial sources while the ones isolated from typed varieties produced the least before and after curing.

Table 4.14: Production of Reducing Sugar (mg/mL) by the selected cured and uncured *L. plantarum* isolates

Isolate code	Reducing sugar Conc. (mg/ml)	
	Uncured	Cured
L. plantarum TV1	*0.742±0.002	0.606±0.002
L. plantarum TV2	0.724±0.002	0.587±0.002
L. plantarum TV3	0.720±0.002	0.564±0.002
L. plantarum LV1	1.174±0.005	0.918±0.005
L. plantarum LV2	1.156±0.005	0.876±0.005
L. plantarum LV3	1.152±0.005	0.888±0.005
L. plantarum C01	0.770±0.003	0.642±0.003
L. plantarum C02	0.760±0.025	0.637±0.025
L. plantarum C03	0.762±0.003	0.635±0.003

*Each value is a mean of duplicate determinations ± Standard Error

Table 4.15: Production of Beta-Galactosidase Enzyme (Unit/mL) By the Selected Cured and Uncured *L. plantarum* isolates

	B-Galactosidase production (unit/mL)	
Isolate code	**Uncured**	**Cured**
L. plantarum TV1	* 0.687±0.002	0.510±0.002
L. plantarum TV2	0.672±0.005	0.512±0.005
L. plantarum TV3	0.674±0.005	0.508±0.005
L. plantarum LV1	0.958±0.005	0.638±0.005
L. plantarum LV2	0.947±0.005	0.635±0.005
L. plantarum LV3	0.940±0.003	0.644±0.003
L. plantarum C01	0.697±0.002	0.518±0.002
L. plantarum C02	0.685±0.002	0.514±0.002
L. plantarum C03	0.683±0.003	0.510±0.003

*Each value is a mean of duplicate determinations ± Standard Error

Figure 4.1a and 4.1b shows the fermentation of the nine isolates in a complex medium in which glucose was substituted with raffinose at different concentration for 48 hrs.

It shows that LV1 was the highest in terms of optical density (0.7); this was followed by LV2 and LV3. The next were C01 (0.65), C02 (0.65) and C03 (0.65) with values that are less than 0.7 the least occurring were TV1, TV2 and TV3 (0.62) respectively.

The difference between the isolates were not noticeable at 48 hrs but at 72 hrs. The highest optical density was observed in LV1, LV23 and LV3 with (0.8) respectively, C01, C02, were the next with 0.7 and TV1, TV2 and TV3 were the unit with 0.6 (This is shown in figure 1b). There was an increase in growth from a concentration of 0.5 mg/ml to 1.5 mg/ml respectively.

The figures (4.2A-4.2C) shows the growth of the isolates using various nitrogen sources; $NaN0_2$, NH_4Cl and Peptone in glucose as carbon source. Using peptone as nitrogen source in glucose LV1, LV2 and LV3 were the highest in optical density with (0.9, 0.8 and 0.8 respectively). This was followed by the typed varieties TV1, and TV2 with (0.7 and 0.7 respectively). The least values were observed in C01 and C02 with 0.6 and 0.6 respectively.

Using $NaN0_2$ as nitrogen sources, the highest values were observed in TV1 with (0.8), this was followed by C01 (0.7) all the least was TV1 (0.6).

There was an increase in growth from a concentration of 0.2 mg/ml to 1.0 mg/ml in all the isolates. NH_4Cl was also used as nitrogen source; TV1 was the highest in which the isolates from typed and commercial varieties were almost the same. Analysis of variance shows a significance difference (p=0.05) in all the nitrogen sources with peptone being the best, this was followed by $NaN0_2$ and NH_4Cl was the least. Peptone favoured the growth of all the isolates in glucose.

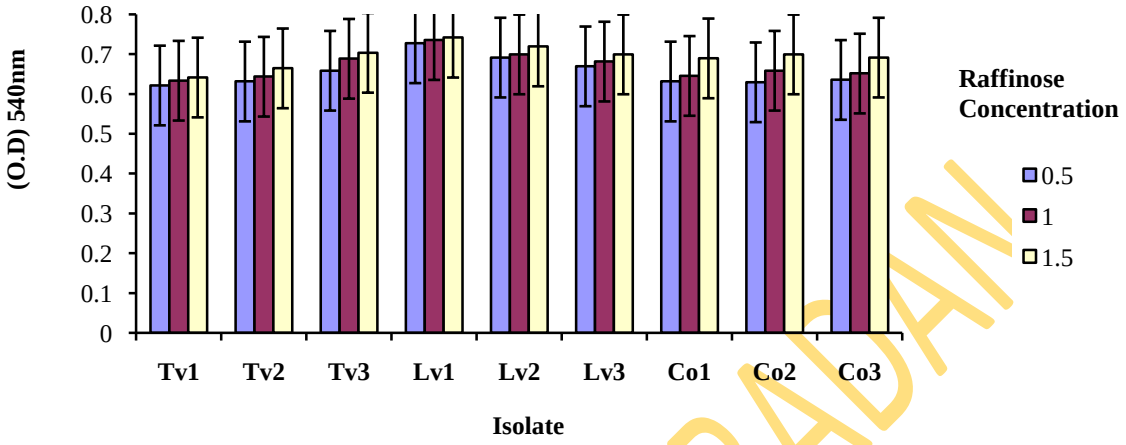

Fig. 4.1a: Fermentation Pattern of Selected *L. plantarum* isolates in a Complex Medium (MRS) substituted with Raffinose for 48 hrs

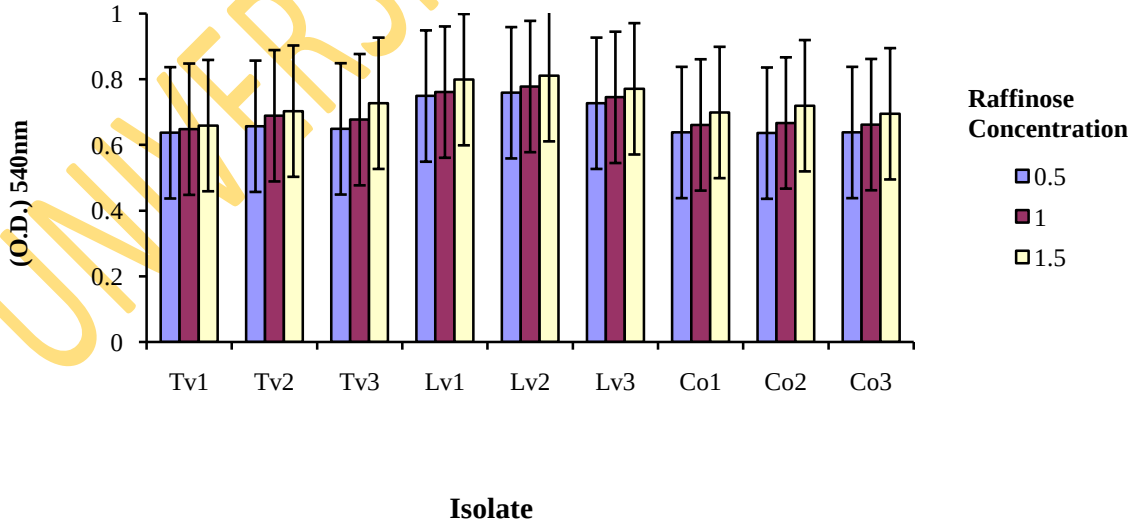

Fig. 4.1b: Fermentation Pattern of Selected *L. plantarum* isolates in a Complex Medium (MRS) substituted with Raffinose for 72 hrs

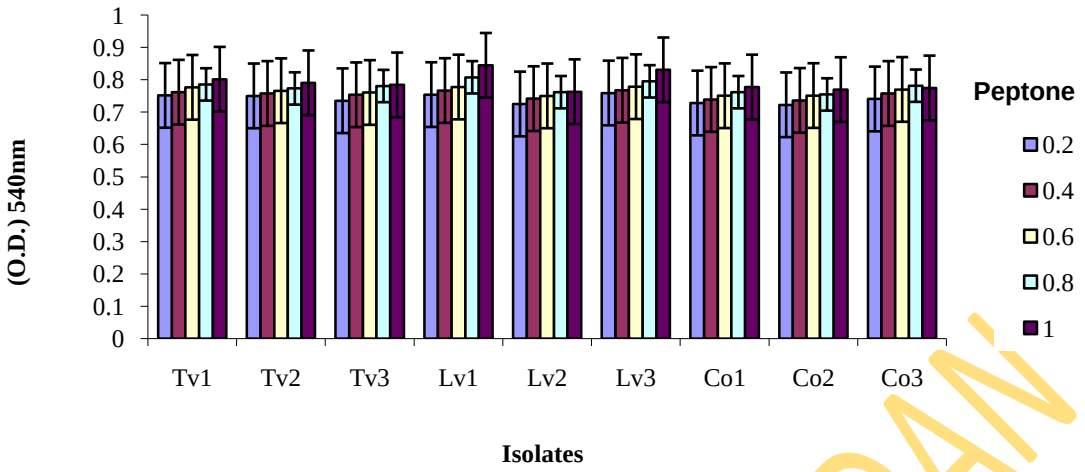

Fig. 4. 2a: Growth in Glucose Using Peptone as Nitrogen Source

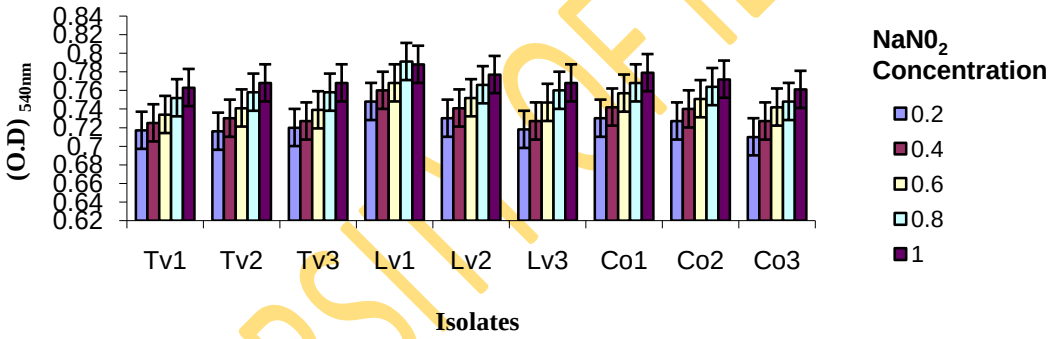

Fig. 4.2b: Growth in Glucose Using $NaNO_2$ as Nitrogen Source

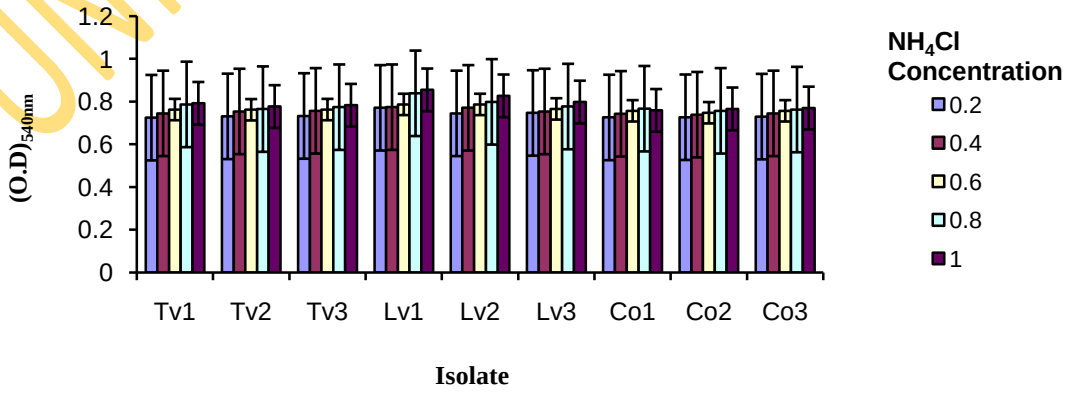

Fig. 4.2c: Growth Using NH_4Cl as Nitrogen Source In Glucose

Figures 4.3A-4.3C shows the growth of the organism when peptone was used as nitrogen source.LV1 and LV2 were the highest, this was followed by TV2 and TV3, the least value was observed in C02 and C03.

Using NH_4Cl as nitrogen source in lactose, LV1, LV2 and LV3 were the highest; this was followed by TV1, TV2 and TV3 while C01, C02 and C03 were the least. There was no significant difference in LV1, LV2 and LV3 and C01, C02 and C03 when $NaN0_2$ was used as nitrogen source. TV1, TV2 and TV3 were the least occurring in terms of growth in lactose. This shows that peptone favoured the growth of the organisms in lactose.

Figures 4.4A-4.4C shows the effect of using peptone as nitrogen source in raffinose. The highest values were obtained in LV1, LV2 and LV3; this was followed by TV1, TV and TV3. The least values were obtained in C01, C02 and C03. $NaN0_2$ favoured the growth of LV1 and LV2 mostly. This was followed by Tv1 and TV2 and the least in C01 and C03.

There was no significance difference ($p=0.05$) when NH_4Cl was used as nitrogen source in raffinose. This shows that peptone favoured the growth of all the isolates than $NaN0_2$ and NH_4Cl. There was a significance difference ($p=0.05$) in the nitrogen sources. The carbon sources showed a significance difference ($p=0.05$) while glucose being the highest, this was followed by lactose and the least was raffinose.

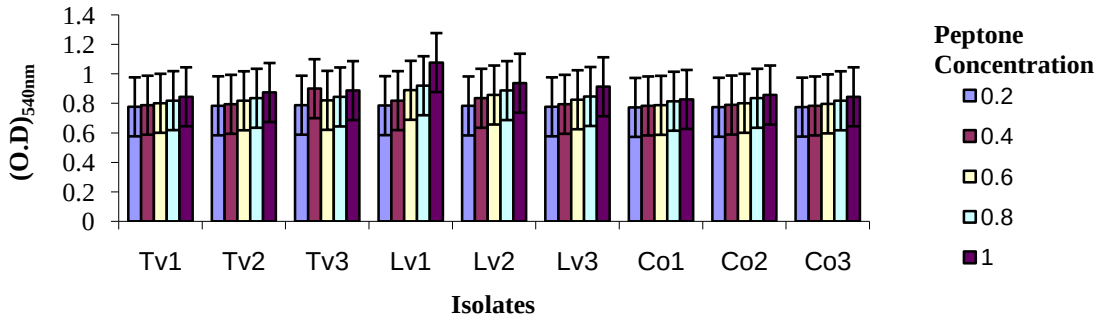

Fig. 4.3a: Growth in Lactose Using Peptone as Nitrogen Source

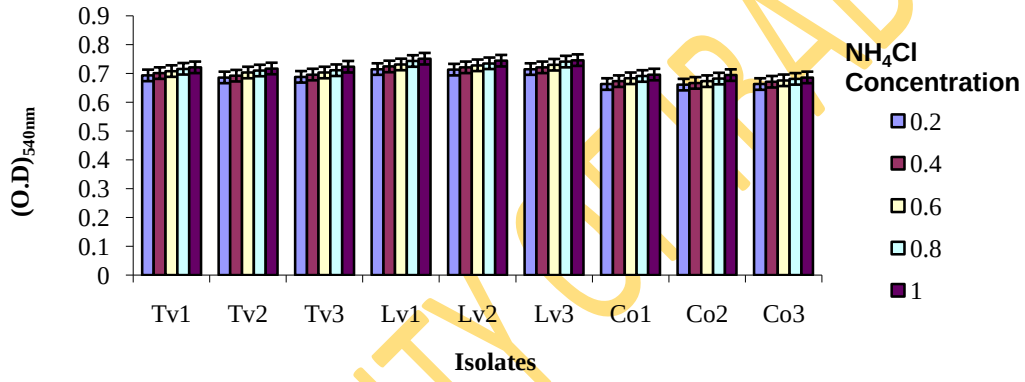

Fig. 4.3b: Growth Using Lactose Using NH_4CL as Nitrogen Source

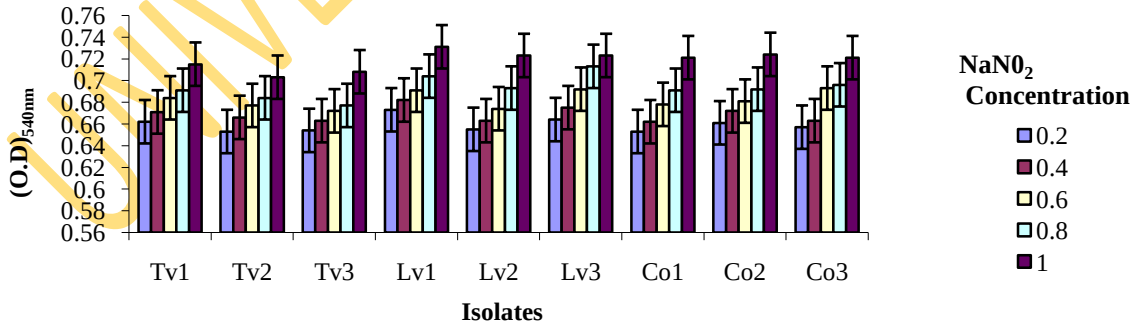

Fig. 4.3c: Growth in Lactose Using $NaNO_2$ as Nitrogen Source

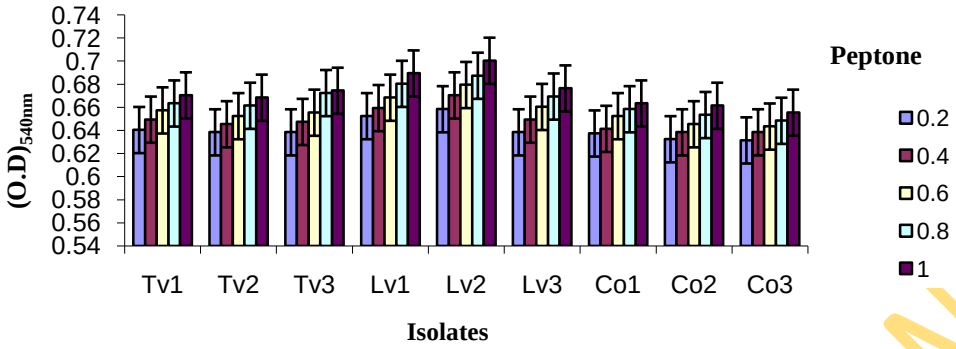

Fig. 4.4a: Growth in Raffinose Using Peptone as Nitrogen Source

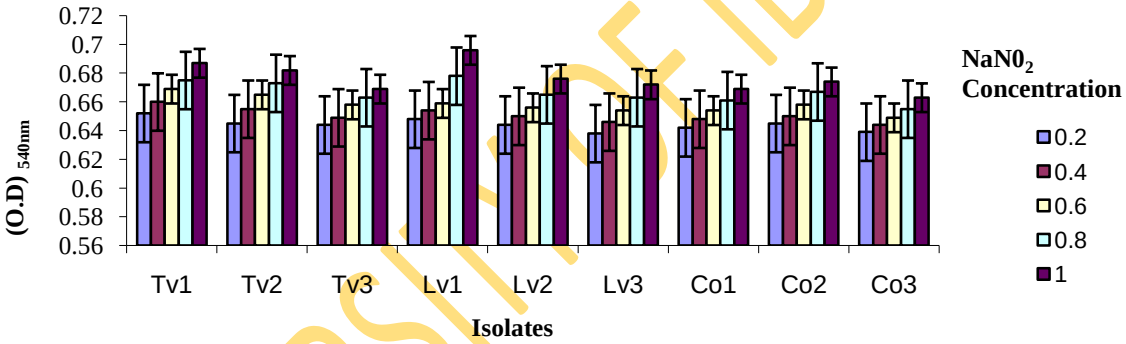

Fig. 4.4b: Growth in Raffinose Using $NaN0_2$ as Nitrogen Source

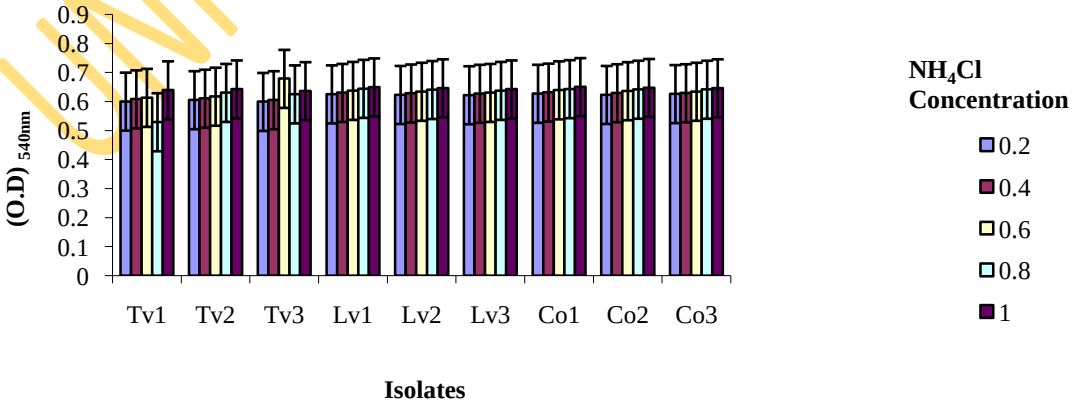

Fig.4. 4c: Growth in Raffinose Using NH_4Cl as Nitrogen Source

The three organisms chosen for further studies were subjected to curing as mentioned earlier. The effect of curing before and after was studied alongside with the various physiological studies.

Figures 4.5, 4. 6 and 4.7 shows the effect of different concentration of glucose, lactose and raffinose on the growth of the selected *L. plantarum*. Glucose favoured the growth of the isolates with optical density (1.2), there was an increase in the growth of the isolates as the sugar concentrations increased. This was followed by lactose with optical density (0.9) and the least was raffinose (0.7). There was a significant difference (p=0.05) in all the sugars, no significant difference was observed among isolates. Curing had a negative effect and *L. plantarum.* The optical density reduced from 0.8 to 0.4 in all the 3 sugars. Curing affected *L. plantarum* significantly.

Figures 4. 8a-4.8c shows the effect of curing on *L. plantarum* when $MgS0_4$ at different concentration was used as the cation source in glucose, lactose and raffinose. There was a significant difference (p=0.05) in all the sugars with glucose being the highest (0.90), this was followed by lactose (0.84) and raffinose the least (0.81). There was increase in growth from a concentration of 0.2 mg/ml – 1.0 mg/ml. Curing affected the growth of the isolates as there was a reduction in the optical density from (0.9) to (0.6). No significant difference (p=0.05) was observed among isolates.

Figures 4.9a-4.9c shows the effect of curing on *L. plantarum* when $ZnS0_4$ at different concentration was used as the cation source in all the sugars. The growth of the organisms varied significantly (p=0.05) with glucose being the highest and raffinose the least. Also, there was an increase in growth from 0.2 mg/ml – 1.0 mg/ml. Curing also had a significant (p=0.05) effect on the growth of *L. plantarum* there was a reduction in growth from 0.88 to 0.52. No significant difference (p=0.05) was observed among isolates.

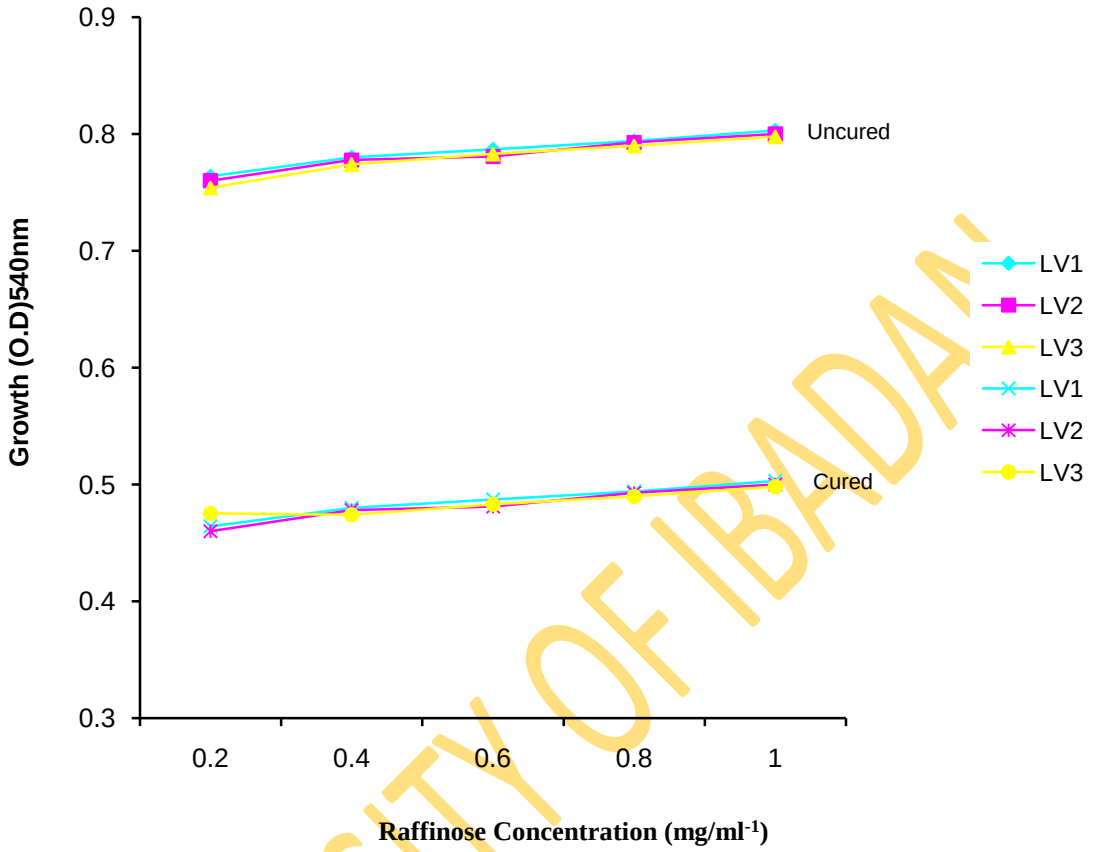

Fig. 4.5: Growth of *L. plantarum* Isolates in Different Concentration of Raffinose

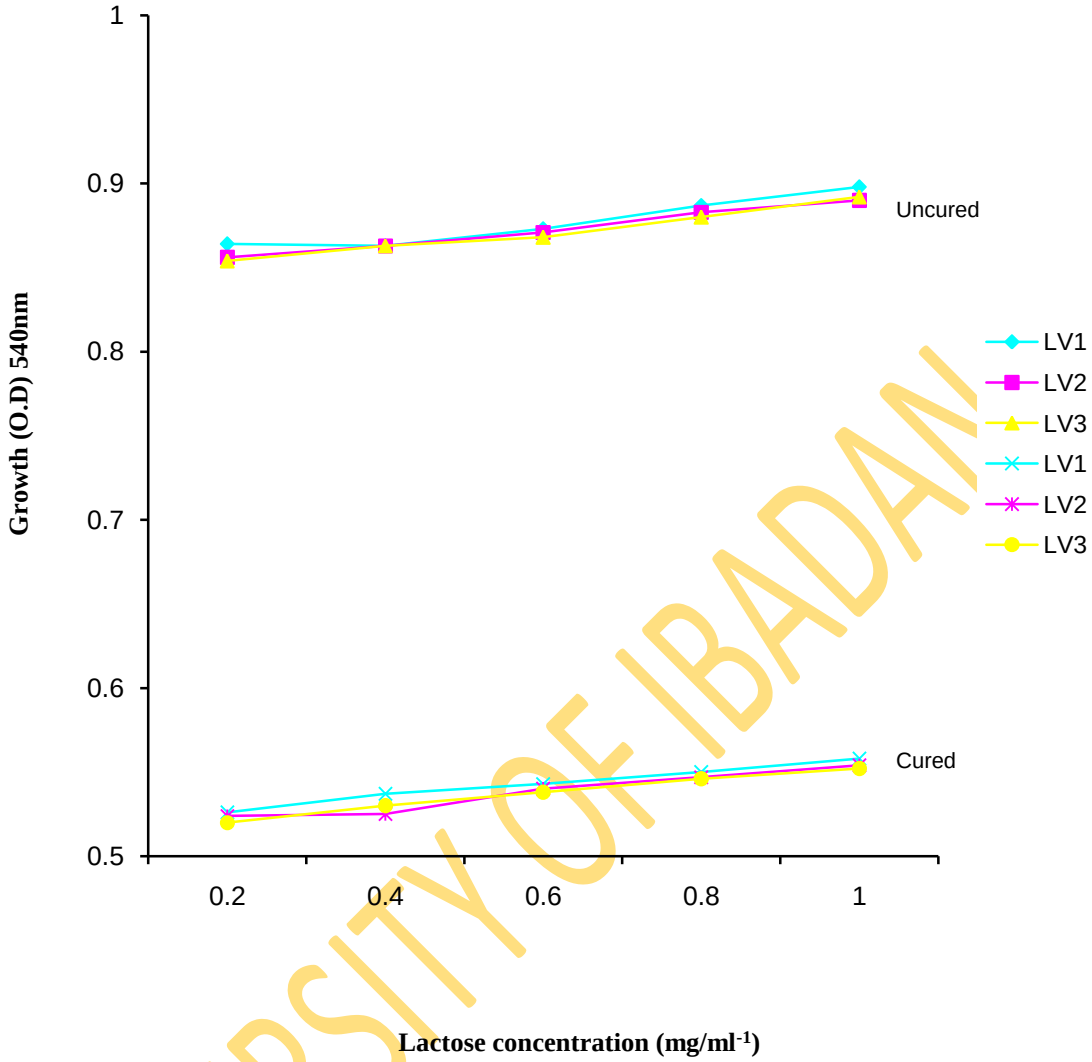

Fig.4. 6: Growth of *L. plantarum* Isolates in different concentration of Lactose

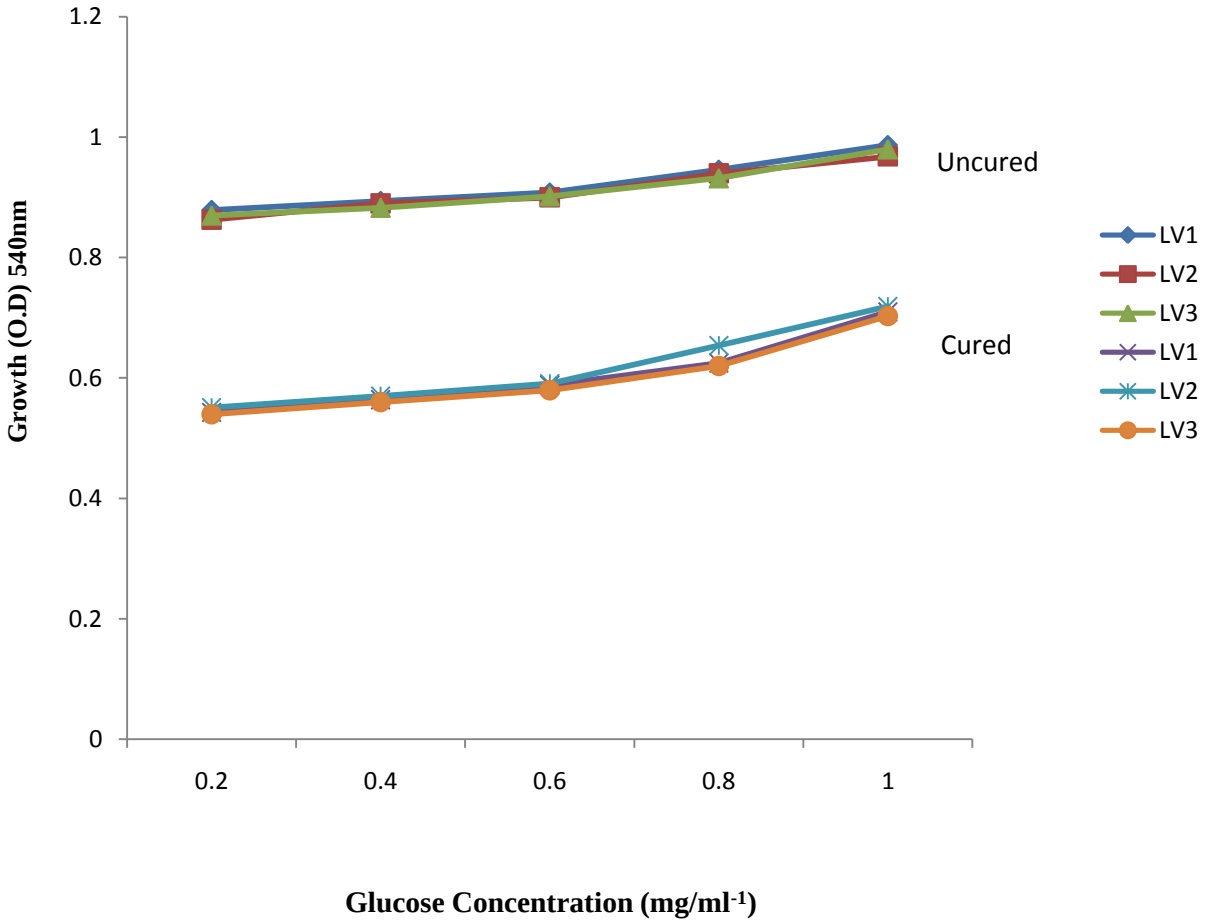

Fig.4.7: Growth of *L. plantarum* Isolates in Different Concentration of Glucose

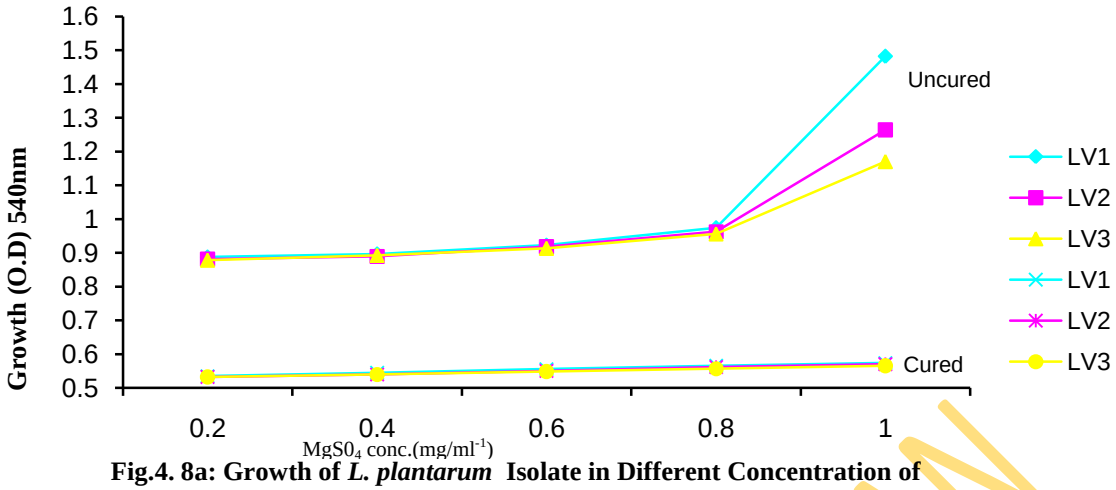

Fig.4. 8a: Growth of *L. plantarum* Isolate in Different Concentration of $MgSO_4$ using glucose as Carbon source

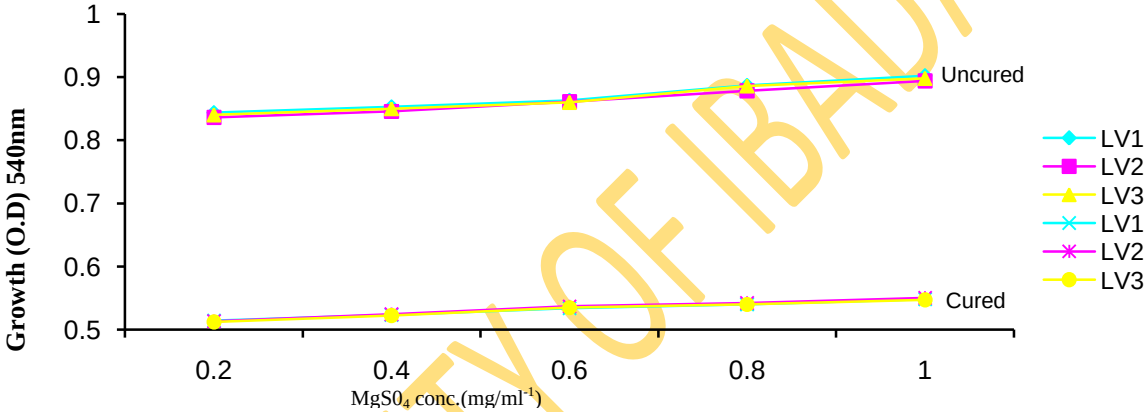

Fig. 4. 8b: Growth of *L. plantarum* Isolate in Different Concentration of $MgS0_4$ Using Lactose as Carbon Source

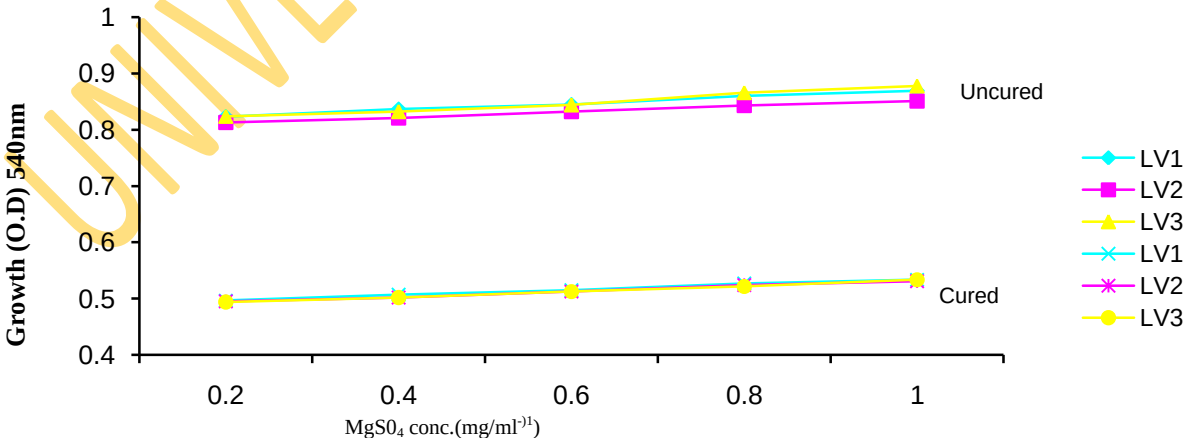

Fig.4. 8c: Growth of *L. plantarum* Isolate in Different Concentration of $MgS0_4$ Using Raffinose as Carbon Source

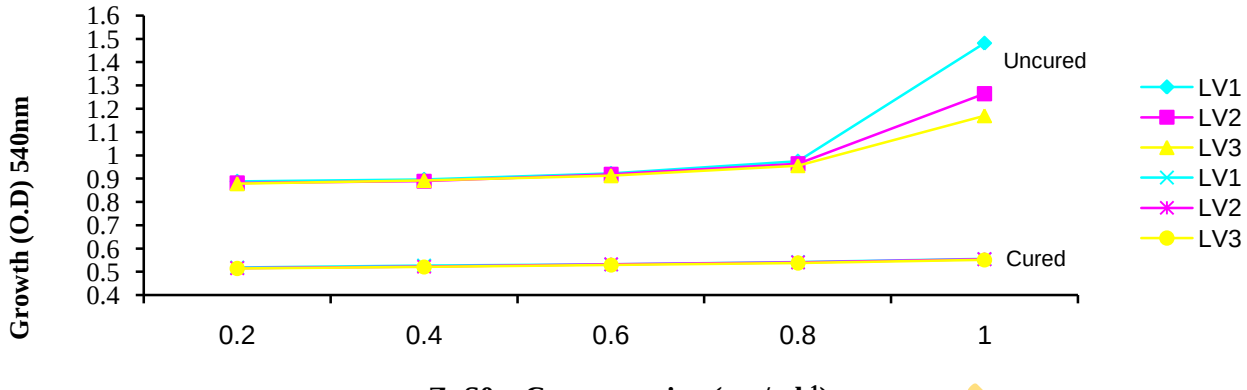

Fig. 4. 9a: Growth of *L. plantarum* Isolate in Different Concentration of ZnS0$_4$ Using Glucose as Carbon Source

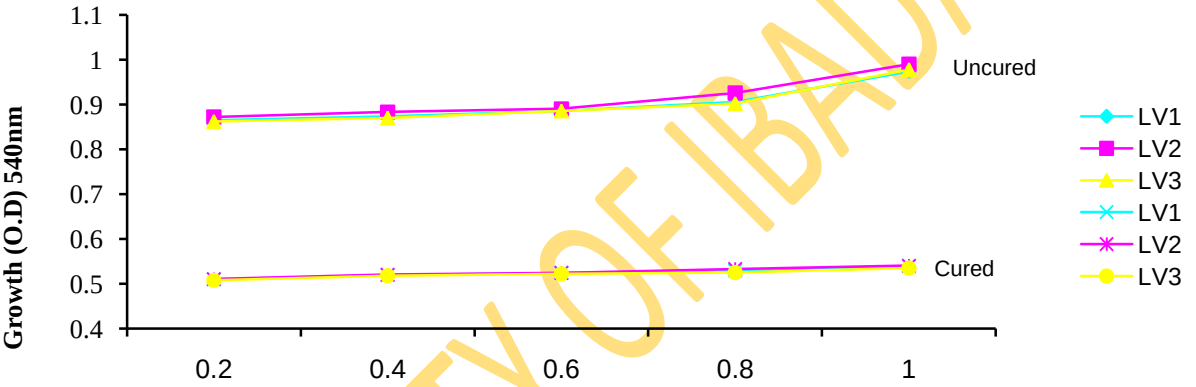

Fig. 4.9b: Growth of *L. plantarum* Isolate in Different Concentration of ZnS0$_4$ Using Lactose as Carbon Source

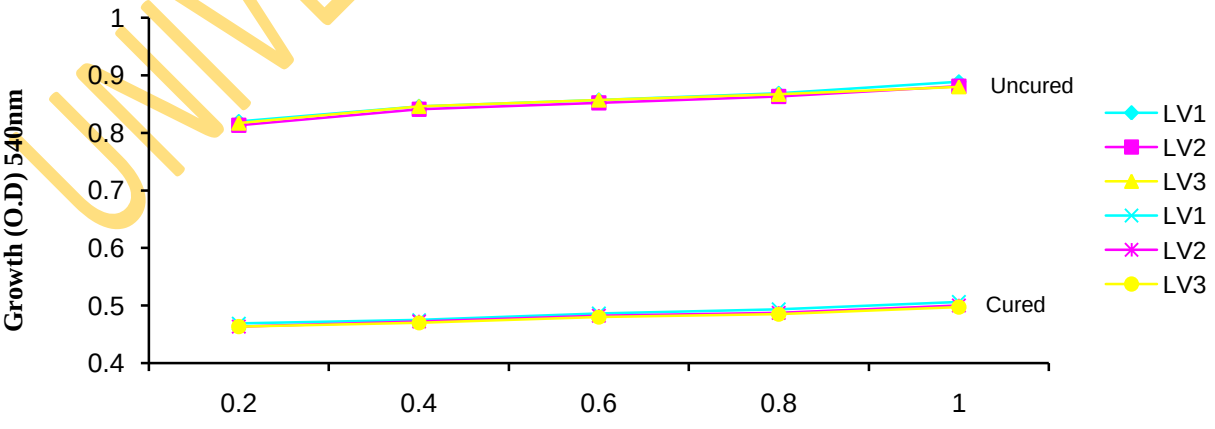

Fig.4. 9c: Growth of *L. plantarum* Isolate in Different Concentration of ZnS0$_4$ Using Raffinose as Carbon Source

Figures 4.10a-4.10c shows the effect of curing on *L.plantarum* when $FeS0_4$ was used as the cation source and the sugars were varied. Glucose shows the highest optical density, this was followed by lactose and raffinose was the least. There was a significant difference (p=0.05) in all the sugar and curing had a negative effect on the growth of *L. plantarum*. From the result also, it could be observed that $MgS0_4$ favoured the growth of *L. plantarum* when compared with other cations. Analysis of variance shows that these was significant difference (p=0.05) in the salts with $MgS0_4$ being the highest (0.92) followed by $FeS0_4$ (0.88) and $ZnS0_4$ was the least (0.82). No significant difference (p=0.05) was observed among isolates.

Figures 4.11a-4.11c Show the effect of curing on the growth of *L. plantarum* when triammonium citrate was used as anion source and the 3 sugars were varied. Glucose shows the highest (1.1), this was followed by lactose (0.9) and the least was raffinose (0.7). There was a significant difference (p=0.05) in all the sugars and curing had a negative effect on the growth of *L. plantarum*. No significant difference (p=0.05) was observed among isolates.

Figure 4.12a-4.12c shows the effect of different concentration of NaCl on the growth of cured and uncured *L. plantarum*. Glucose favours growth most (0.9), followed by lactose (0.7) and the least was raffinose (0.6). There was significant difference (p=0.05) in all the sugars. Curing also affected the growth of the isolates in NaCl and there was a significant difference (p=0.05) in growth between the cured and uncured isolates. No significant difference (p=0.05) was observed among isolates.

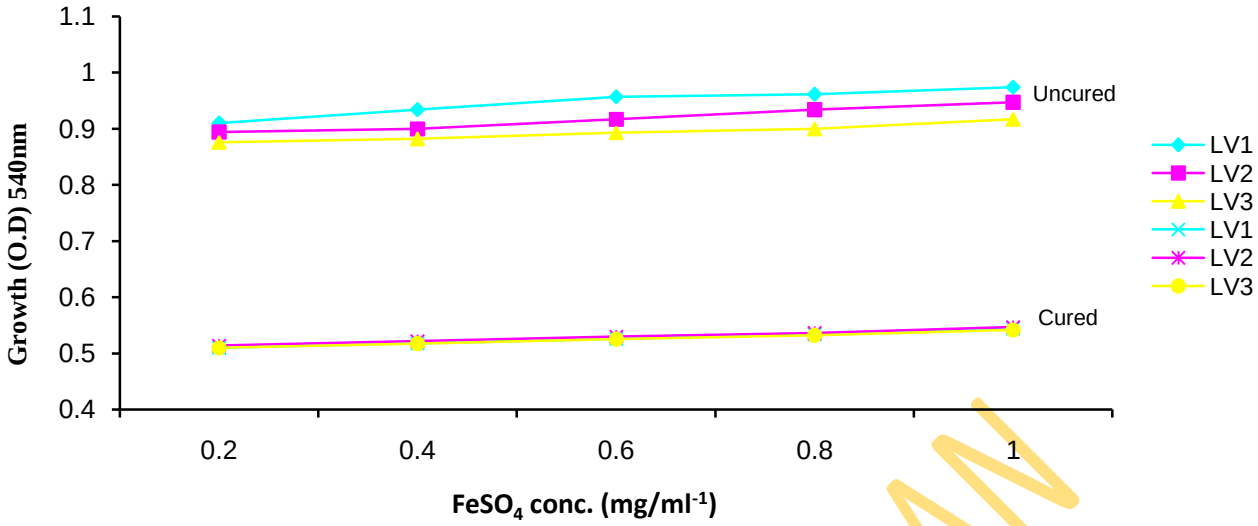

Fig.4. 10a: Growth of *L. plantarum* Isolates in Different Concentration of $FeS0_4$ Using Glucose as Carbon Source

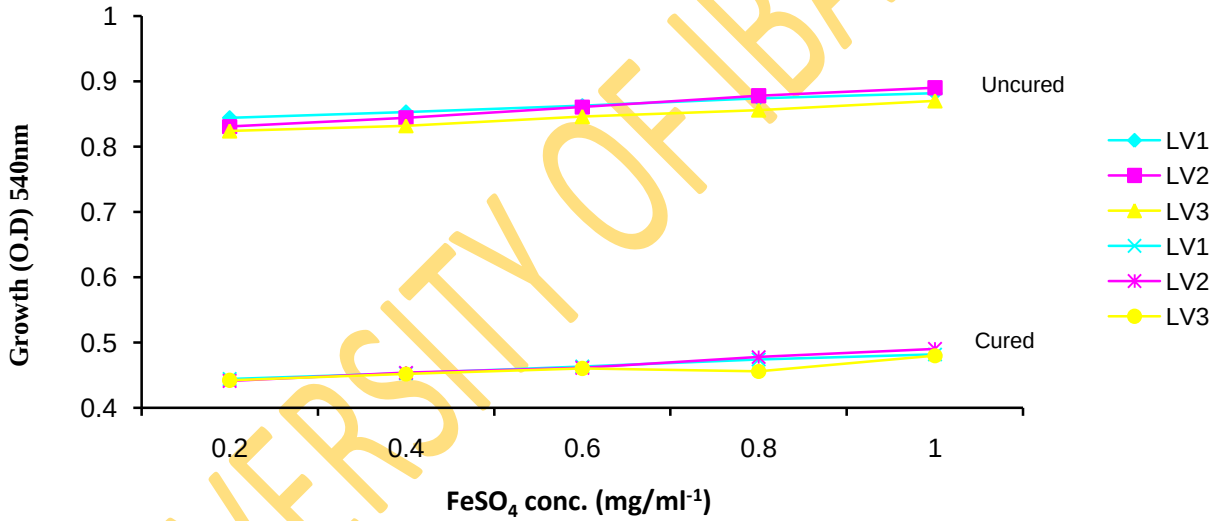

Fig.4.10b: Growth of *L. plantarum* Isolates in Different Concentration of $FeS0_4$ Using Lactose as Carbon Source

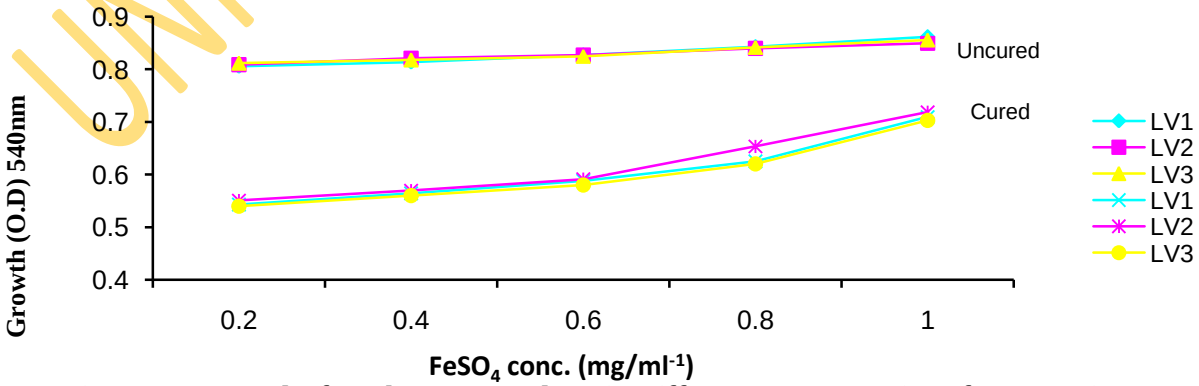

Fig. 4.10c: Growth of *L. plantarum* Isolates in Different Concentration of $FeS0_4$ Using Raffinose as Carbon Source

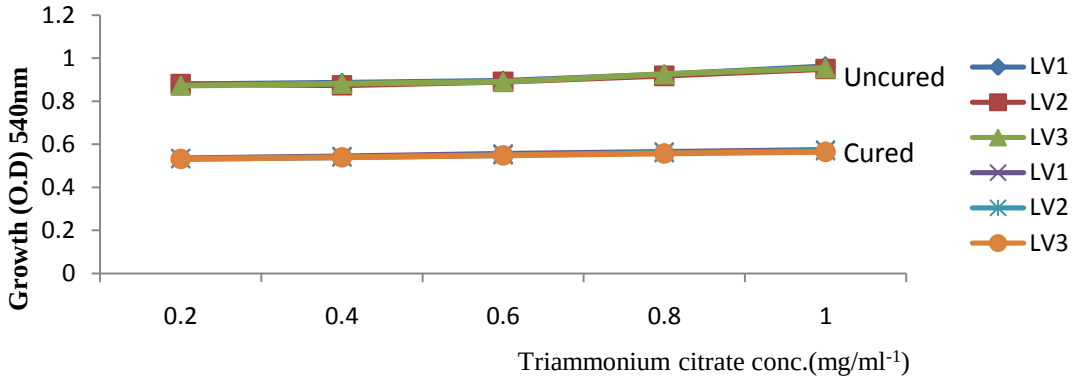

Fig. 4.11a : Growth of *L. plantarum* Isolate in Different Concentration of Triammonium Citrate Using Glucose as Carbon Source

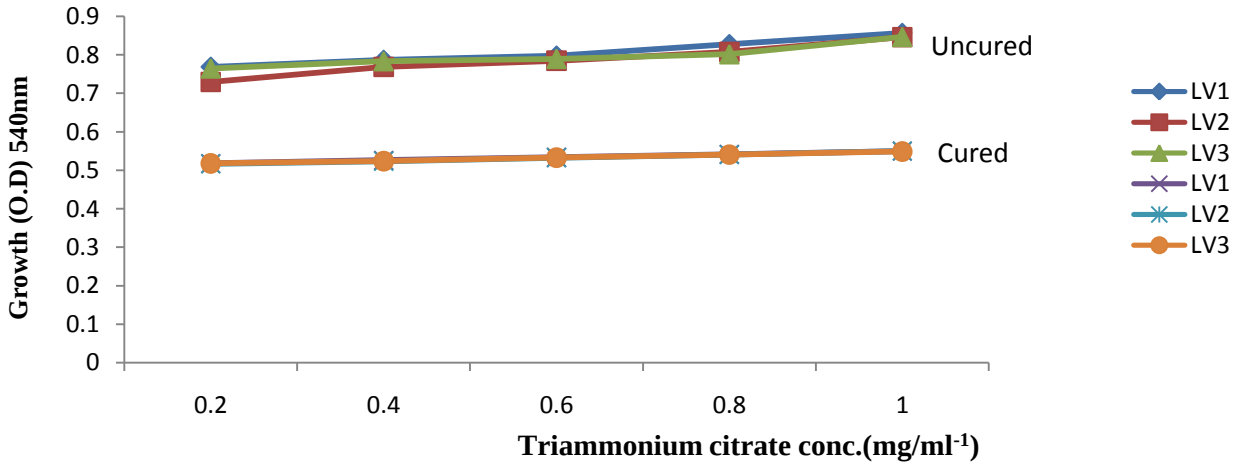

Fig. 4.11b : Growth of *L. plantarum* Isolate in Different Concentration of Triammonium Citrate using Lactose as Carbon Source

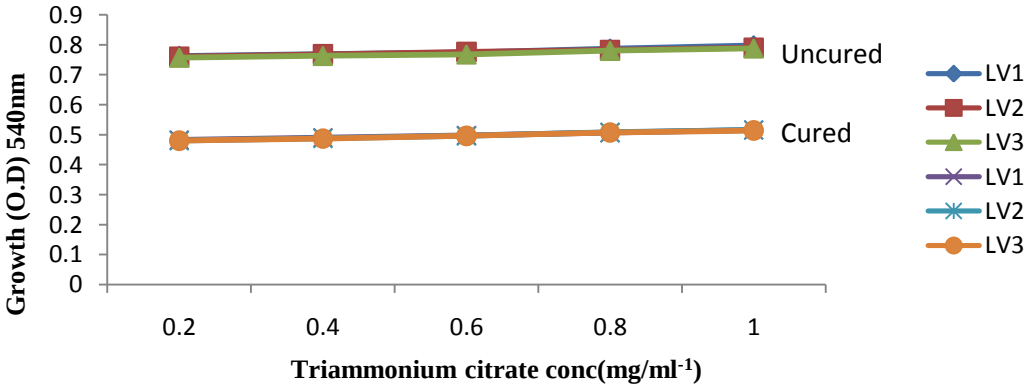

Fig.4. 11c: Growth of *L. plantarum* Isolate in Different Concentration of NaCl Using Lactose as Carbon Source

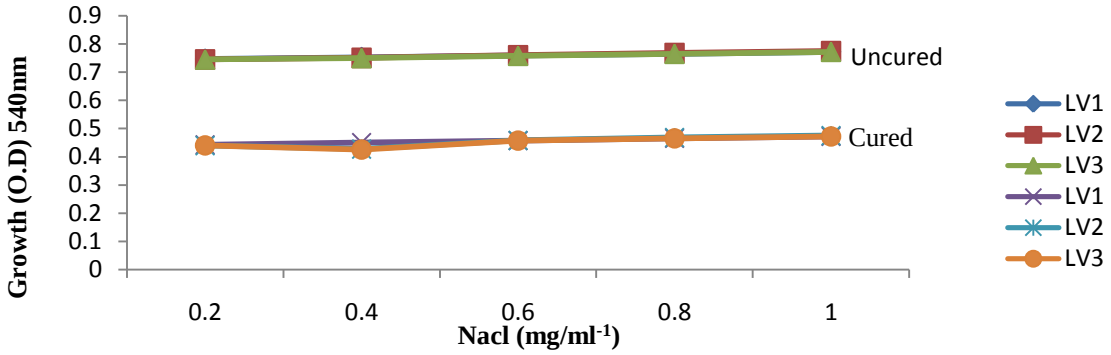

Fig. 4.12a: Growth of *L. plantarum* Isolate in Different Concentration of NaCl Using Glucose as Carbon Source

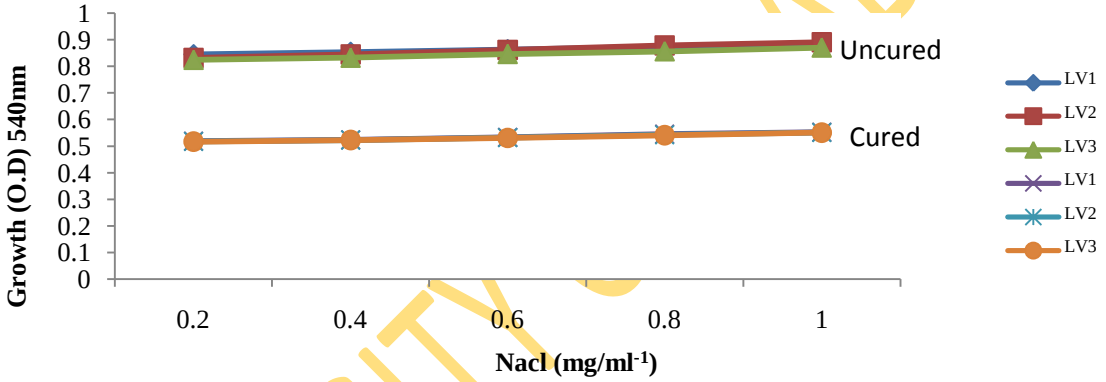

Fig. 4. 12b: Growth of *L. plantarum* Isolate in Different Concentration of NaCl Using Lactose as Carbon Source

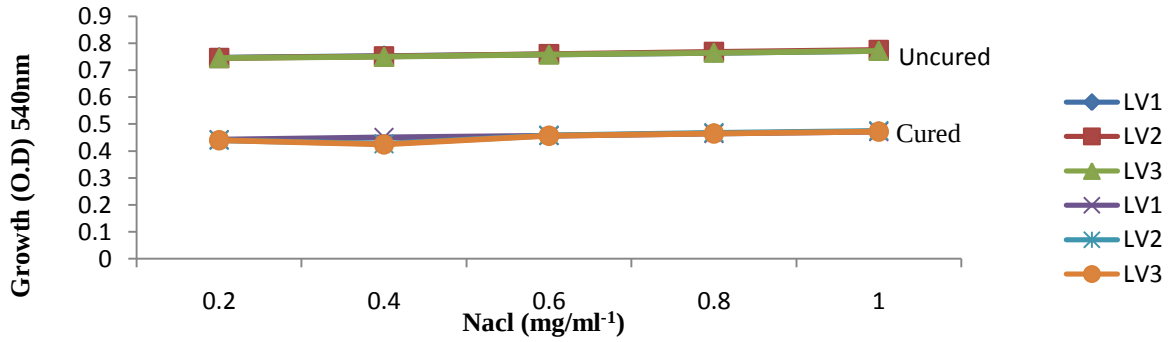

Fig. 4.12c: Growth of *L. plantarum* Isolate in Different Concentration of NaCl Using Raffinose as Carbon Source

Figures 4.13a- 4.13c shows the effect of different concentration of $MgCl_2$ on the growth of cured and uncured *L. plantarum* glucose favours growth (0.9) more than lactose (0.7) and raffinose (0.6). There was a significance difference (p=0.05) in the result obtained for growth in all the sugars and the cured and uncured *L. plantarum*. Effect of different concentrations of anions on *L. plantarum* shows that traiammonium citrate favours growth most (1.2) then NaCl (1.0) and $MgCl_2$(0.9). There was a significant difference (p=0.05) in all the anions used in the medium of growth. No significant difference (p=0.05) was observed among isolates.

Figures4.14a-4.14c shows the effect of different temperature ranges on the growth of cured and uncured *L.plantarum.* The growth reached the peak at 30^0C, this is the optimum. Growth reduced significantly (p=0.05) at a rather high (60^0C) or low temperature (20^0C).Glucose favours growth most when compared to other sugars, there was significant difference (p=0.05) in the growth using different sugars. Plasmid curing affected the growth of the isolate significantly. No significant difference (p=0.05) was observed among isolates.

Figures 4.15a-4.15c shows the effect of different pH ranges on the growth of cured and uncured *L.plantarum.* A pH of 5.5 favoured growth most, this is the optimum. At a higher pH (9), there was a reduction in growth. Also, at a lower pH (3), there was reduction in growth. Glucose shows a significant increase (p=0.05) in growth than other sugars. There was a reduction in the growth of the isolate when the cured isolates were used compared to the uncured isolates. No significant difference (p=0.05) was observed among isolates.

Figure 4.16a-4.16c shows the effect of different time interval on the growth of cured and uncured *L.plantarum* isolates. As the incubation time increases, there was an increase in growth. The peak was at 72hrs, there was a slight reduction in growth at 96hrs and 120 hrs. Glucose favours the growth of the isolates than all the other sugars. Plasmid curing affected the growth f the organisms. The reduction in growth at 96 hrs and 120 hrs was significant (p=0.05) with the cured isolates. No significant difference (p=0.05) was observed among isolates.

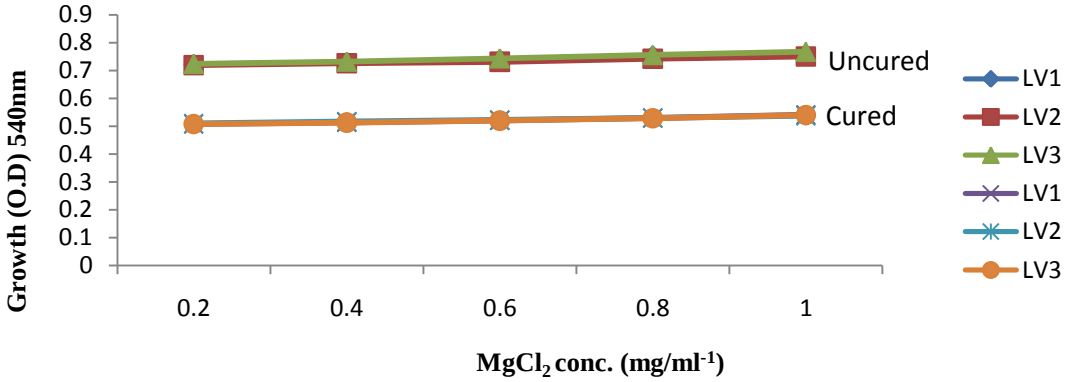

Fig. 4.13a: Growth of *L. plantarum* Isolate in Different Concentration of $MgCl_2$ Using Glucose as Carbon Source

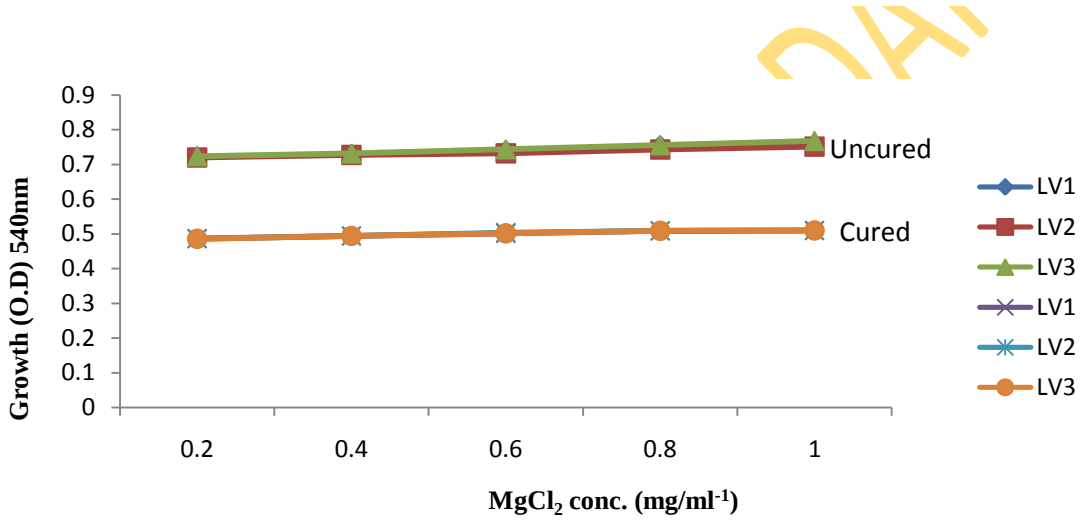

Fig. 4. 13b: Growth of *L. plantarum* Isolate in Different Concentration of $MgCl_2$ Using Lactose as Carbon Source

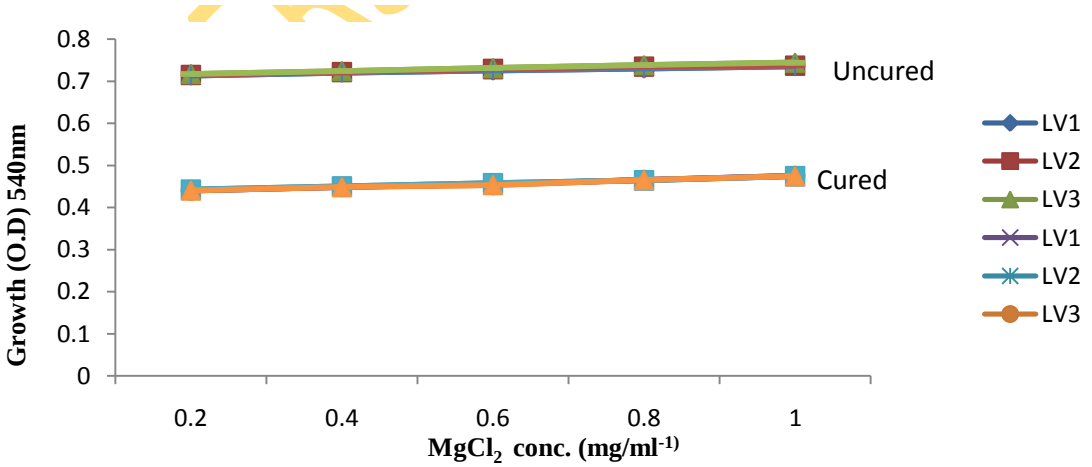

Fig. 4. 13c: Growth of *L. plantarum* Isolate in Different Concentration of $MgCl_2$ Using Raffinose as Carbon Source

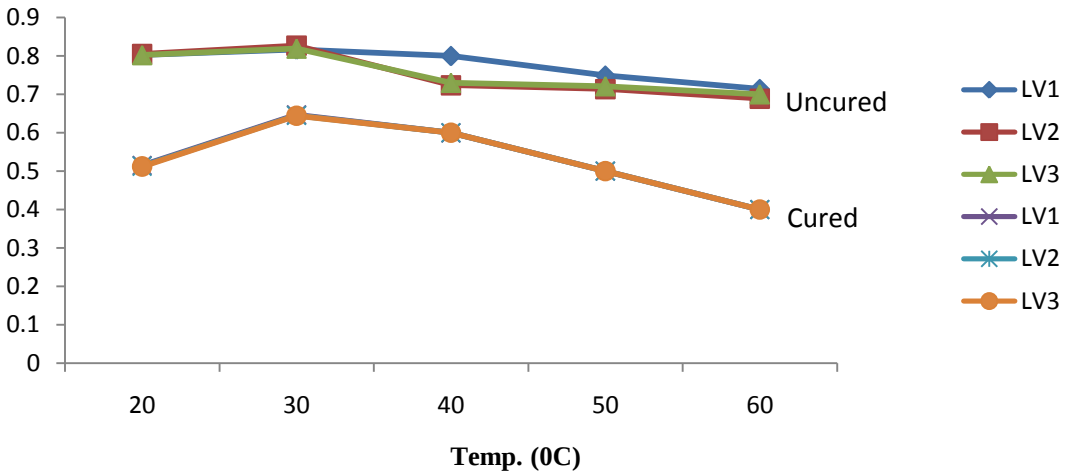

Fig. 4. 14c: Growth of *L. plantarum* Isolate at Different Temperature ranges using Raffinose as Carbon Source

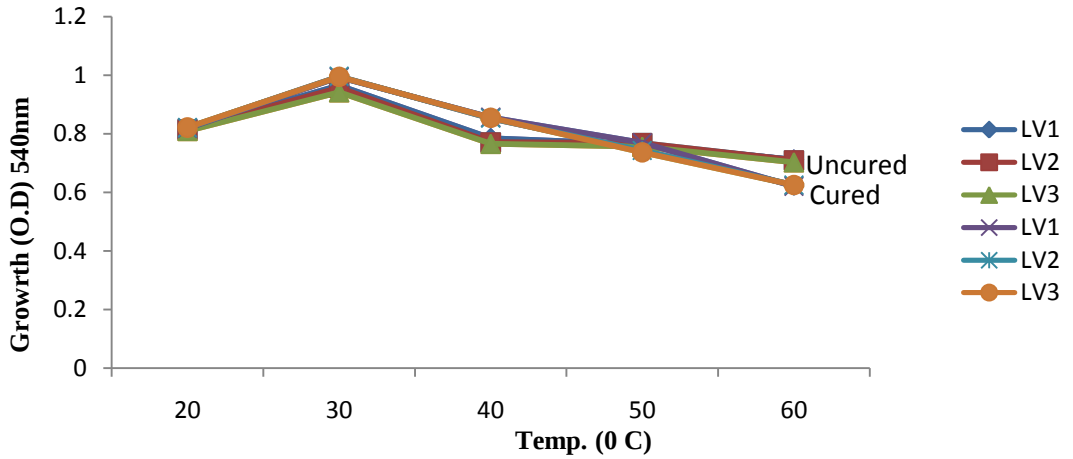

Fig.4. 14a: Growth of *L. plantarum* Isolate at Different Temperature ranges Using Glucose as Carbon Source

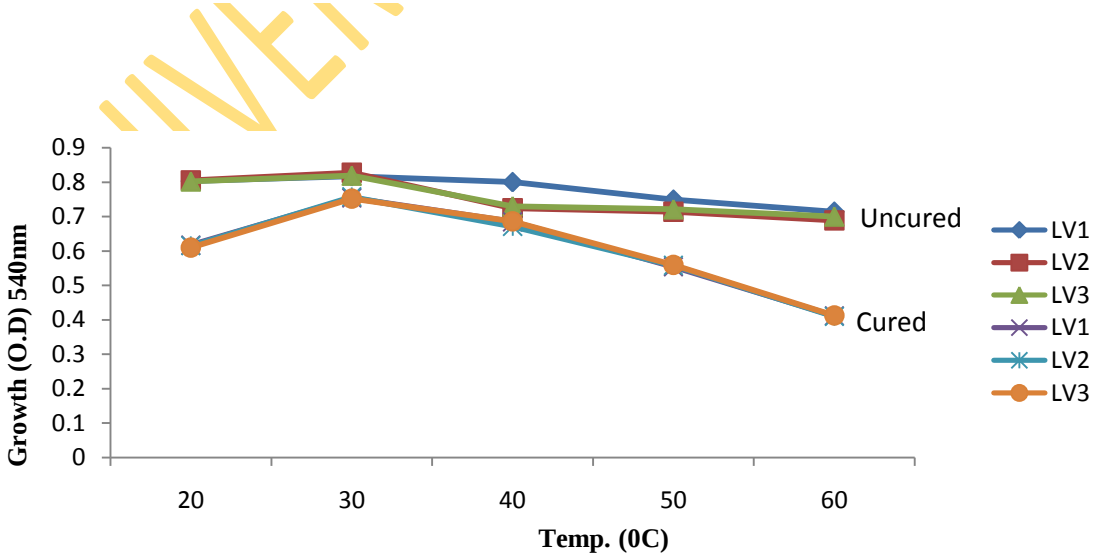

Fig.4. 14b: Growth of *L. plantarum* Isolate at Different Temperature ranges Using Lactose as Carbon Source

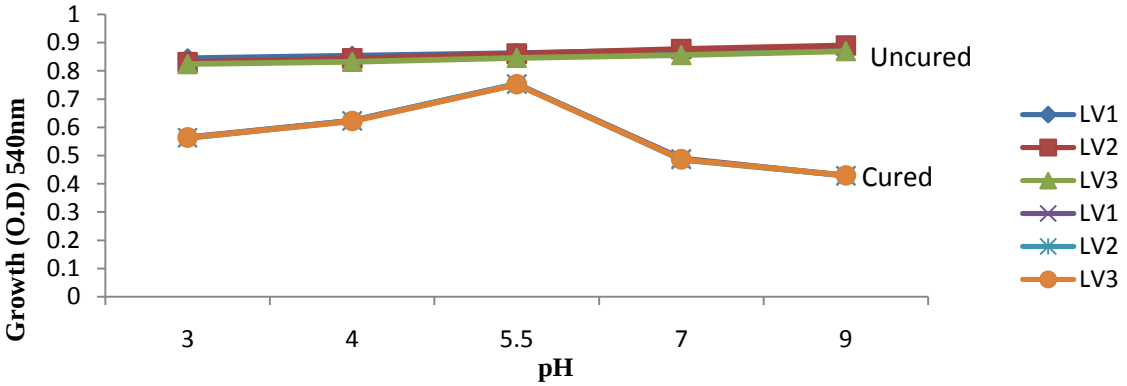

Fig.4. 15a: Growth of *L. plantarum* Isolate at Different pH ranges Using Glucose as Carbon Source

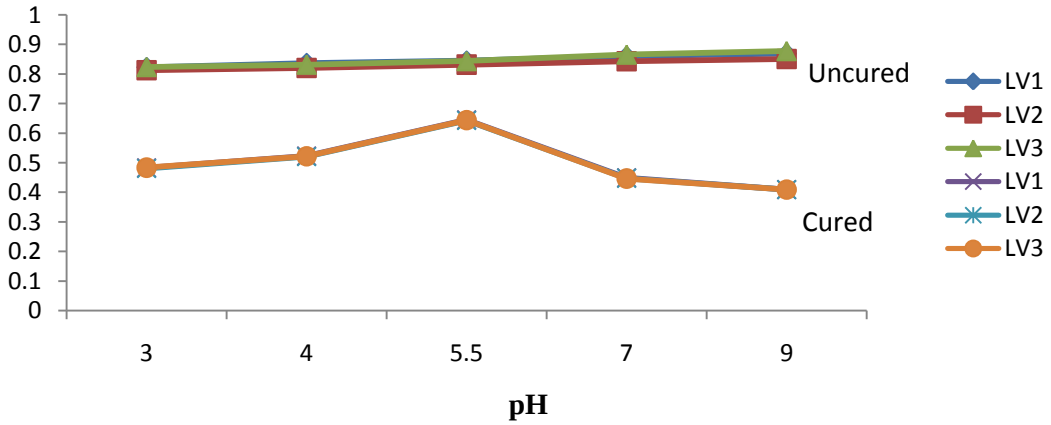

Fig.4.15b: Growth of *L. plantarum* Isolate at Different pH ranges Using Lactose as Carbon Source

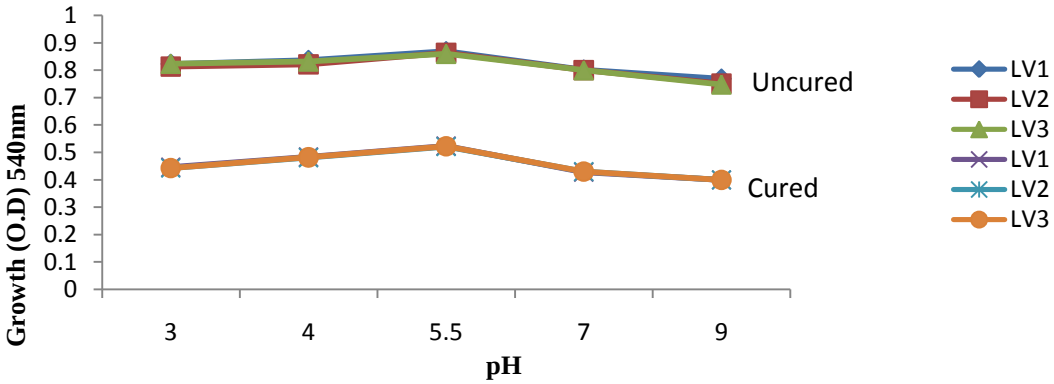

Fig.4. 15c: Growth of *L. plantarum* Isolate at Different pH ranges Using Raffinose as Carbon Source

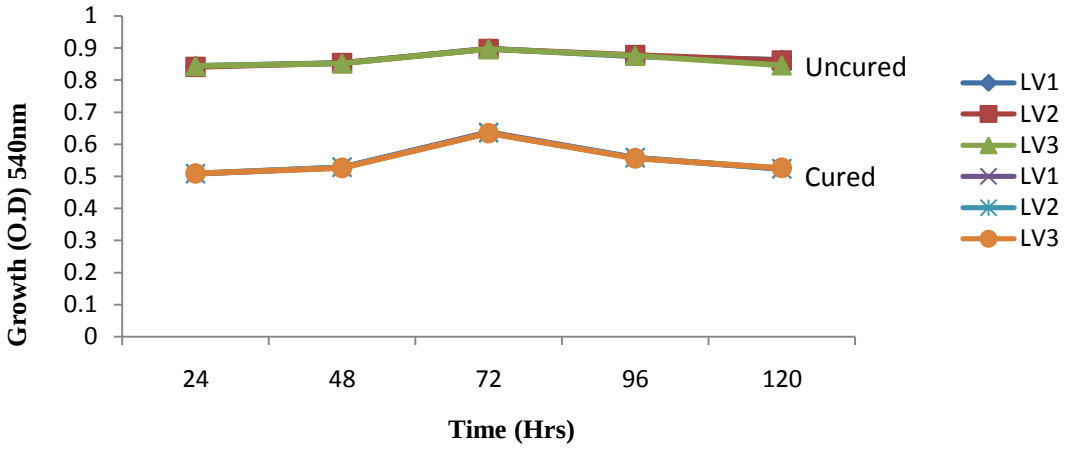

Fig.4. 16a: Growth of *L. plantarum* Isolate at Different Time Interval Using Glucose as Carbon Source

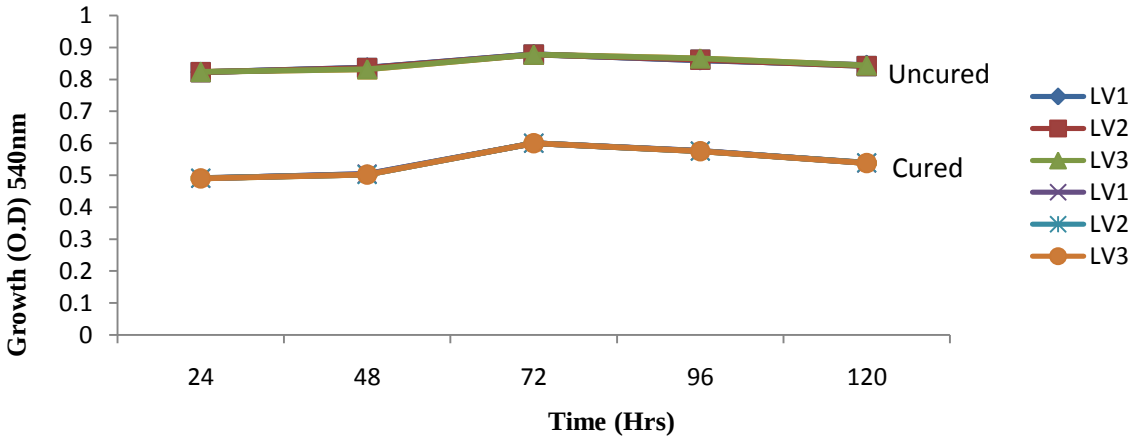

Fig.4. 16b: Growth of *L. plantarum* Isolate at Different Time Interval Using Lactose as Carbon Source

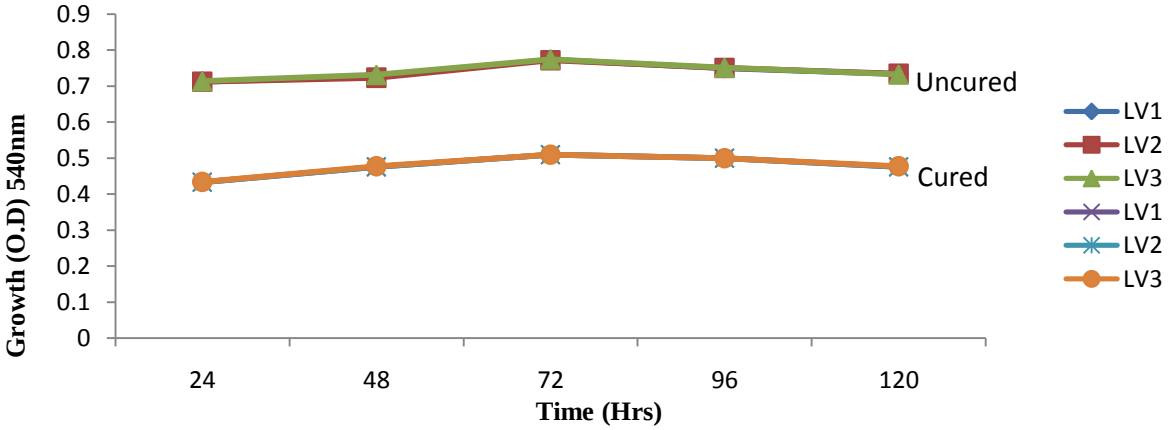

Fig.4. 16c: Growth of *L. plantarum* Isolate at Different Time Interval Using Raffinose as Carbon

As observed from the figures above, curing affected all the physiological characteristics of *L. plantarum* significantly (p=0.05). It affected the growth and the ability of the organisms to utilize various cations, anions and different carbon sources for growth significantly (p=0.05). It also affected the pH, temperature and time interval of growth significantly (p=0.05).

From the physiological characteristics of the isolates result above, the organism, *L.plantarum* utilized the various sugars in different ways, glucose being the one that was utilized most, and this was followed by lactose, while raffinose was the least. Curing also affected the ability of the organism to utilize various sugars. After curing of the organisms, the ability of the various organisms to utilize the various sugars reduced, the organisms could not metabolise the sugars like before. Curing had a negative effect on the ability of the isolate to metabolise glucose, lactose and raffinose for growth at various concentrations.

Table 4.16 shows the result of the ten topmost sequences producing significant alignments when the nucleotide sequences were subjected to Basic Local Alignment Search Tool (BLAST) in the gene bank Database (http://www.ncbi.nlm.nih.gov/blast/Blast.igi) for *L.plantarum* isolates.

Altogether, ten *L.plantarum* isolates which has been identified before using conventional methods showed a significant alignment in the gene database. The names and accession number have significant alignments with the *L.plantarum.* All the ten topmost species was shown to produce significant alignment with the marker and have the expected (E value) of between 1e-7e and 5e-7 and maximum identification (Max identity) of between 95% and100%. They were all *Lactobacillus plantarum.*

Table 4.17 show the Qualities and quantities of the 16S rDNA genes of the *L.plantarum* obtained by PCR using V3 primer, after purification.

Figure 4.21 shows the trees generated by BLAST pair wise alignment tool; it revealed the degree of relatedness of the 16S rDNA gene nucleotide sequences of the test *L.plantarum* to those in the gene database. The identified *L.plantarum* isolates had more closeness to *Lactobacillus plantarum* with the accession number.

|GQ180905.1|*Lactobacillus plantarum* strain TJ2 16S ribosomal RNA gene, partial sequence, Length=182, Score = 283 bits (153), Expect = 1e-73,

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Identities = 153/153 (100%), Gaps = 0/153 (0%) Strand=Plus/Plus

Query       GTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTT
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Query       AAAGAAGAACATATCTGAGAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCA
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct       AAAGAAGAACATATCTGAGAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCA

Query       CGGCTAACTACGTGCCAGCAGCCGCGGTAATAA
            |||||||||||||||||||||||||||||||||
Sbjct       CGGCTAACTACGTGCCAGCAGCCGCGGTAATAA
```

|GQ180906.1|*Lactobacillus plantarum* strain DGTJ3 16S ribosomal RNA gene, partial sequence Length=181, Score = 281 bits (152), Expect = 5e-73

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 Identities = 152/152 (100%), Gaps = 0/152 (0%), Strand=Plus/Plus

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Sbjct       AAAGAAGAACATATCTGAGAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCA

Query       CGGCTAACTACGTGCCAGCAGCCGCGGTAATA
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|GQ166663.1|*Lactobacillus plantarum* strain LpT2 16S ribosomal RNA gene, partial sequence, Length=1469, Score = 281 bits (152), Expect = 5e-73

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Sbjct  357  GTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTT

Query  63   AAAGAAGAACATATCTGAGAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCA
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Sbjct  417  AAAGAAGAACATATCTGAGAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCA

Query  123  CGGCTAACTACGTGCCAGCAGCCGCGGTAATA
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Sbjct  477  CGGCTAACTACGTGCCAGCAGCCGCGGTAATA
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|GQ166662.1|*Lactobacillus plantarum* strain LpT1 16S ribosomal RNA gene, partial sequence, Length=1481, Score = 281 bits (152), Expect = 5e-73

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```

```
Sbjct       GTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTT

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Sbjct       AAAGAAGAACATATCTGAGAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCA

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```

```
|FJ861114.1|Lactobacillus plantarum strain KLDS 1.0630 16S ribosomal RNA
gene, partial sequence, Length=1450, Score =  281 bits (152),  Expect =
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Sbjct       GTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTT

Query       AAAGAAGAACATATCTGAGAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCA
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Sbjct       AAAGAAGAACATATCTGAGAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCA

Query       CGGCTAACTACGTGCCAGCAGCCGCGGTAATA
            |||||||||||||||||||||||||||||||
Sbjct       CGGCTAACTACGTGCCAGCAGCCGCGGTAATA
```

Figure.4. 17: Alignment of 16s rDNA nucleotide sequences of *L. plantarum* against *Lactobacillus plantarum* strain LpT2 (accession no GQ166663.1) and *Lactobacillus plantarum* strain LpT1 (accession no GQ166662.1) in the gene bank data base

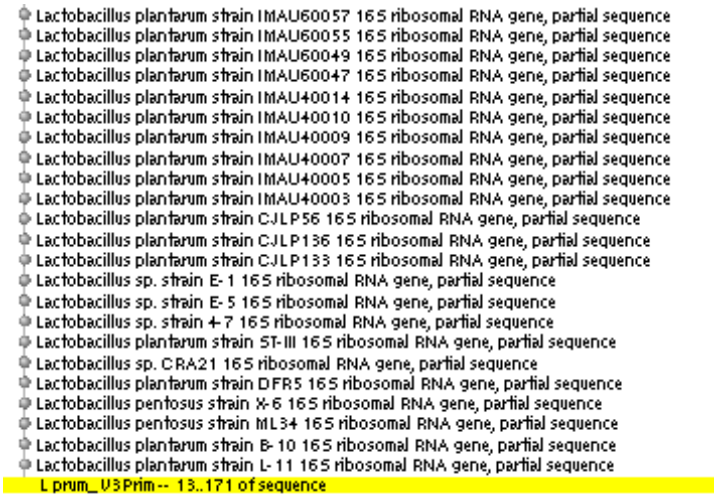

Figure 4.18: The trees generated by BLAST pairwise alignment tool.

Table 4.16 shows the result of the ten topmost sequences producing significant alignments when the nucleotide sequences were subjected to Basic Local Alignment Search Tool (BLAST) in the gene bank Database (http://www.ncbi.nlm.nih.gov/blast/Blast.igi) for *L. plantarum* isolates.
Altogether, 10 *L. plantarum* isolates that have been identified before showed a significant alignment in the gene database. The names and accession numbers have significant alignments with the *L. plantarum*. All the ten topmost species was shown to produce significant alignment with the marker and have expected value (E value) of between 1e – 73 and 5e – 7 and maximum identification (Max identity) of between 95% and 100%. They were all *Lactobacillus plantarum*.

Table 4.17 below shows the Qualities and quantities of the 16S rDNA genes of the *L. plantarum* obtained by PCR using V3 primer, after purification.

Figure 4. 20 below shows the PCR products of 16s V3 rDNA of the LAB isolate, BLA1, resolved in 2.0% Agarose after electrophoresis at 84 volts for 1.5-2.0 hr in 1X TAE buffer. Molecular characterisation of the isolates was done by extracting the DNA and the bands shown after running them on 10% agarose gel. This is shown in figure 20.
The DNA was later used in other molecular analysis of the *L. plantarum* isolates.

Figure 4.21 shows the molecular weight of the plasmids of the selected nine *L.plantarum* isolate that were used for further work.
The figure shows the marker labelled M and the other ten isolates. The first nine isolates are the nine that were selected because they contain and produced plasmids. Some other organisms were screened which did not show the presence of plasmids. The production of plasmids by the nine isolates confers a greatest advantage on them. This was harnessed when they were used for further studies. The bands showed the production of plasmids by the isolates on the gel.

Figure 4.22 shows the plasmid picture after the curing of the nine *L. plantarum* isolates. It could be observed that it was the sample labelled M which is the marker that only showed the band and the molecular weight of the marker. The other ten isolates bands did not show the plasmid, this is because it has been cured/ destroyed. Once the plasmid has been cured, the bands will no longer show in the gel.

Table 4.16 :Identities of *L.plantarum* and sequenced identities

Isolate code	Conventional identities	Closest relative	% Identity	Gene bank accession
L.plantarum C01	*L. plantarum*	*L. plantarum*	**95%**	**GQ180906.1**
L.plantarum C02	*L. plantarum*	*L. plantarum*	**95%**	**GQ166663.1**
L.plantarum C03	*L. plantarum*	*L. plantarum*	**95%**	**GQ166662.1**
L.plantarum LV1	*L. plantarum*	*L. plantarum*	**95%**	**FJ861114.1**
L.plantarum LV2	*L. plantarum*	*L. plantarum*	**95%**	**FJ861113.1**
L.plantarum LV3	*L. plantarum*	*L. plantarum*	**95%**	**FJ861112.1**
L.plantarum TV1	*L. plantarum*	*L. plantarum*	**95%**	**FJ84495 .1**
L.plantarum TV2	*L. plantarum*	*L. plantarum*	**95%**	**FJ844955.1**
L.plantarum TV3	*L. plantarum*	*L. plantarum*	**95%**	**FJ844949.1**

Table 4. 17: Qualities and quantities of the 16S rDNA genes of the *L. plantarum* obtained by PCR using V3 primer, after purification

S/N	Sample ID	16S rDNA Conc. (ug/L)	A260nm	A260/280	0.0260/230
5	H101	18.34	0.367	1.82	0.02

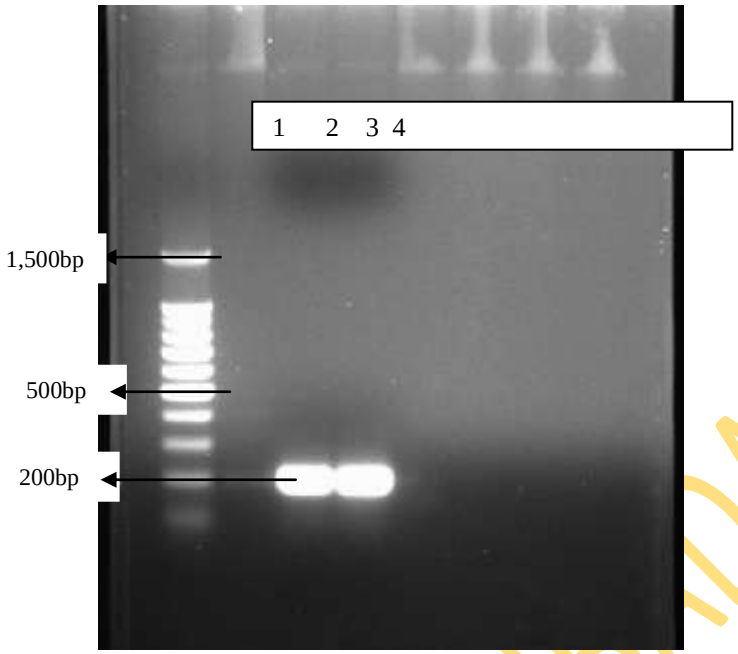

Figure 4.20: PCR products of 16s V3 rDNA of the LAB isolate, BLA1, resolved in 2.0% Agarose after electrophoresis at 84 volts for 1.5-2.0 hr in 1X TAE buffer

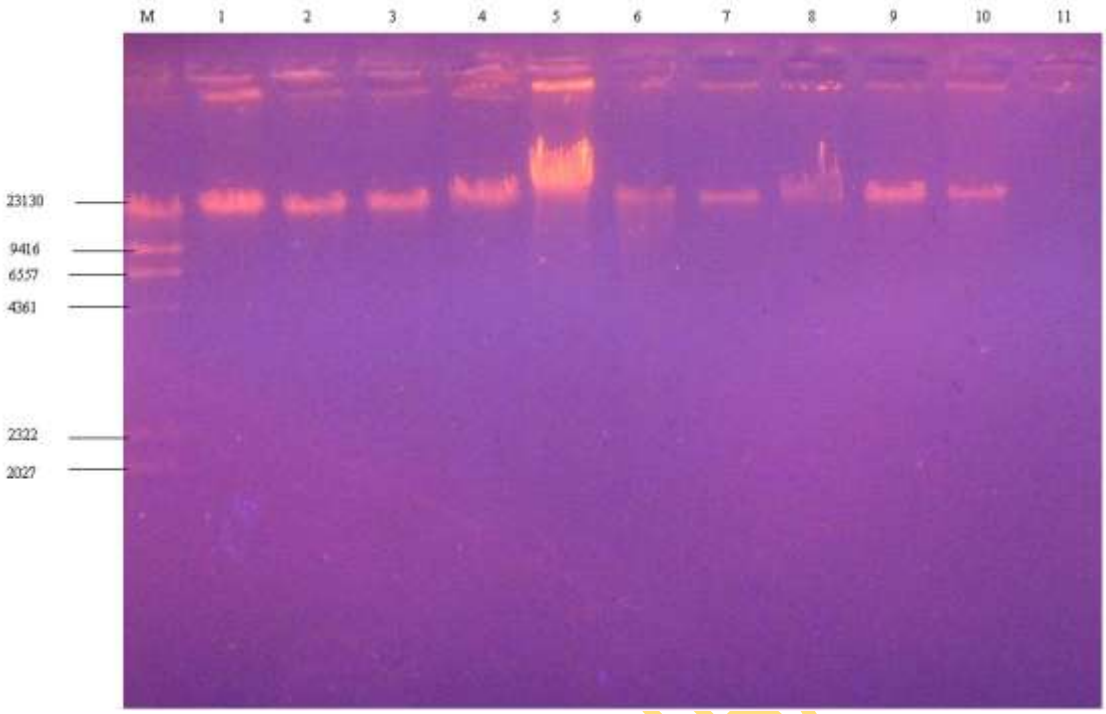

Figure 4.21: Plasmid Picture of selected nine *L.plantarum* isolates before Curing

Key:
M= Marker
1= *L. plantarum* TV1
2= *L. plantarum*TV2
3= *L. plantarum*TV3
4= *L. plantarum* LV1
5= *L. plantarum* LV2
6= *L. plantarum* LV3
7=*L. plantarum* C01
8= *L. plantarum* C02
9= *L. plantarum* C03
10= *L. plantarum*LS10
11= *L. plantarum* LS11

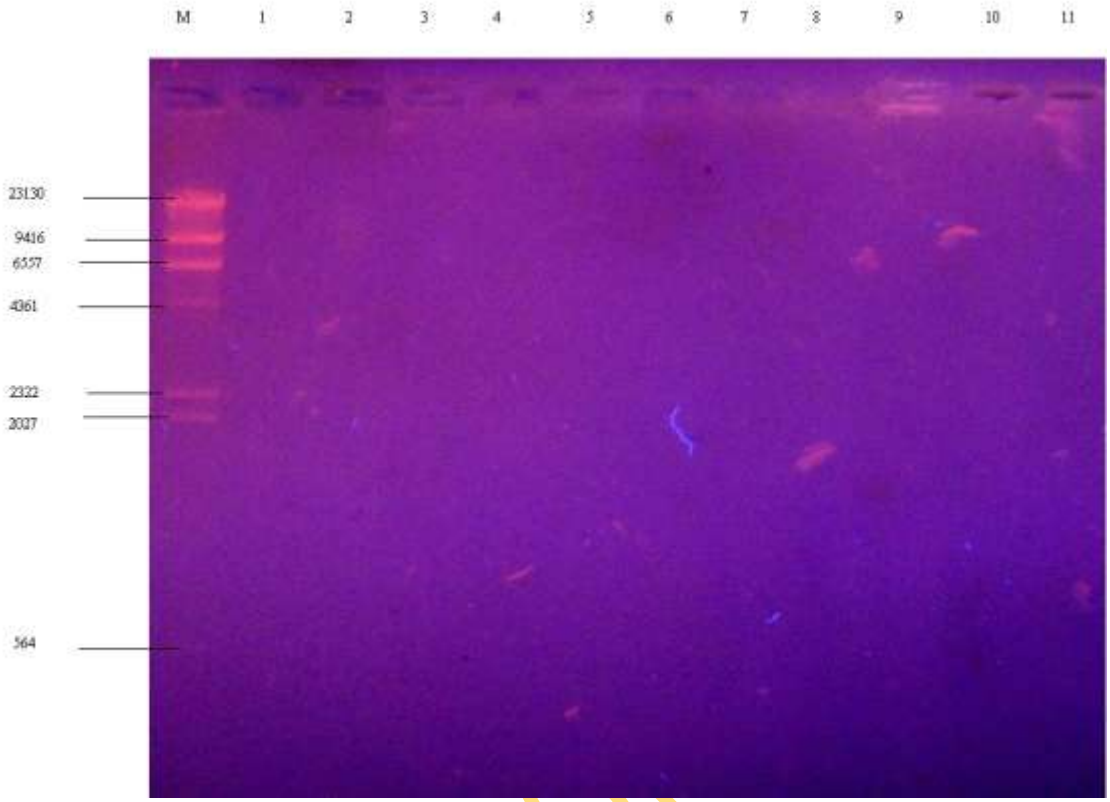

Figure4.22: Plasmid Profile of selected nine *L. plantarum* isolates after Curing

Key:
M= Marker
1= *L. plantarum* TV1
2= *L. plantarum*TV2
3= *L. plantarum*TV3
4= *L. plantarum* LV1
5= *L. plantarum* LV2
6= *L. plantarum* LV3
7=*L. plantarum* C01
8= *L. plantarum* C02
9= *L. plantarum* C03
10= *L. plantarum*LS10
11= *L. plantarum* LS11

Table 4.18 shows the Reduction of Anti-nutrients in Soybean during pre-treatment and fermentation. Soybeans contain ANFs like Tannin, Phytate, Trypsin and Protease inhibitor. It was pre-treated by cooking and roasting. It was observed that roasting reduced the different anti-nutritional factors better than other pre-treatment methods. The raw soybeans contain tannin 1.93 mg/g, it was reduced to 1.12 by cooking for while roasting reduced it to 0.49 mg/g. Roasting also reduced the phytate content from 1.6 to 0.25 mg/g, trypsin inhibitor from 1.20 to 0.025 mg/g, and protease inhibitor from 1.20 to 0.03 mg/g. Cooking reduced the anti-nutritional factor but not as much as roasting. There was a significant difference between the pre-treatment methods.

Fermentation also reduced the antinutritional factors in the samples a great deal, the roasted sample was chosen for further work using uncured *L. plantarum* isolates from all the pre-treated methods. Fermentation reduced the tannin content in the raw sample from 1.93 to 0.12 mg/g. Phytate content reduced from 1.16 to 0.04 mg/g. The trypsin inhibitor and protease inhibitor reduced from 1.20 to 1.07 and 1.2 to 1.08 respectively.

There was thus a significant difference in the pre-treatment methods and fermentation. Using cured *L. plantarum* isolate a little significant difference was observed in the various ANFs. The values obtained only reduced a little when compared with the raw samples. Tannin reduced from 1.9 to 1.5, phytate reduced from 1.1 to 1.0, trypsin inhibitor from 1.2 to 1.1 and protease inhibitor from 1.2 to 1.1. A little change was observed when the cured isolates were used. Curing has a significant effect on the reduction of ANFs only reduced a little uncured *L. plantarum* isolate is therefore a better organism when it comes to the reduction of ANFs.

From the result below, it could be observed that fermentation with uncured

L .plantarum isolates reduced the antinutritional factor in the food blend than the samples fermented with cured *L. plantarum* isolate. Also, the different pre-treatment methods affected the antinutritional factors before curing in different ways. Roasting the soybeans before curing reduced all the antinutritional factors significantly than cooking of the sample; this is shown when compared with the raw soybeans samples.

Table 4.18: Anti-nutritional (mg/g) factors in the soybeans blend *'ogi'* after the pre-treatment of the Samples and fermentation for 5 days

A.N.F	Raw (Before fermentation)	Cooked	Roasted	Fermented With Uncured Isolates	Fermented With Cured Isolates
Tannin	1.93 ± 0.19a	1.12 ± 0.02c	0.49 ± 0.12d	0.12 ± 0.05e	1.52 ± 0.17b
Phytate	1.16 ± 0.05a	0.28 ± 0.02c	0.25 ± 0.03d	0.047 ± 0.03e	1.02 ± 0.03b
Trypsin Inhibitor	1.20 ± 0.12a	0.05 ± 0.05c	0.02 ± 0.25d	0.010 ± 0.02e	1.07 ± 0.12b
Protease Inhibitor	1.20 ± 0.02a	0.05 ± 0.05c	0.03 ± 0.03d	0.020 ± 0.03e	1.08 ± 0.02b

*Each value is a mean of duplicate determinations with Standard Error

Table 4.19 below the table shows the nutritional analysis of the raw, roasted, cooked and fermented soybeans samples. From a preliminary study that was done earlier, the roasted soybean was chosen for the fermentation studies. This is because roasting reduced the ANF's better than all pre-treatment methods used.

It will be observed that fermentation with uncured *L. plantarum* increased and improved the nutritional composition of the soybeans. Fermentation increased the Ascorbic acid in mg/100g composition from 6.5 in roasted sample to 16.3 while there was no increase when the cured *L. plantarum* was used. The reducing sugar in % also increased from 0.4 to 0.6, thiamine mg/100g increased from 0.4 to 0.8, riboflavin mg/100g from 0.2 to 0.8, niacin mg/100g from 1.6 to 2.7 while there was no increase when the cured *L. plantarum* was used.

The mineral composition of the soybeans also increased. Ca^{2+} (mg/100g) increased from 170 to 180, Fe^{2+} (mg/100g) from 4.3 to 4.9 while phosphate (mg/100g) increase from 275 to 285. There was no increase when the cured *L. plantarum* was used.

The roasted sample was chosen for further work because the pre-treatment method reduced the oligosaccharide content to the barest minimum when compared to other pre-treatment methods.

Table 4.20 shows the nutritional analysis of the different varieties of *Ogi* that were used for isolation – White maize, Yellow maize and Sorghum samples. It was observed that the nutritional composition of the *Ogi* made from sorghum was more. The protein content was 3.9% in *Ogi* from sorghum while the one from white maize was 1.6% and the one from yellow maize 1.8%. The crude fibre was 1.7% in sorghum and 1.3 in white and yellow maize respectively. The riboflavin content (mg/100g) was 0.10 in sorghum and 0.05 and 0.05 in white and yellow maize respectively. Fe^{2+} content in mg/100g was 4.2 in sorghum and 2.2 and 2.6 in white and yellow maize respectively. The phosphate content also follows the same trend.

Reference was made to this because the *ogi* made from sorghum was chosen for the composition of the weaning food blend. This is because the nutritional content of this sample is more and it explains why in various weaning food composition, sorghum is usually preferred.

Table 4.19: Nutritional Analysis of Soybean Samples before and after fermentation with cured and uncured *L.plantarum*

Parameters	Raw	Roasted	Cooked	Roasted and Fermented with uncured *L. plantarum*	Roasted and Fermented with cured *L. plantarum*
Moisture Content %	*11.2 ± 0.02	8.7 ± 0.14	48.8 ± 0.20	42.1 ± 0.20	45.2± 0.12
Protein %	30.5 ± 0.25	31.8 ± 0.10	17.3 ± 0.10	33.2 ± 0.20	31.9+ 0.10
Ether Extract (Fat) %	22.1 ± 0.10	23.4 ± 0.25	12.9 ± 0.03	14.1 ± 0.20	10.1± 0.20
Ash %	4.3 ± 0.02	4.5 ± 0.03	2.7 ± 0.25	1.3 ± 0.02	1.0± 0.20
Crude fibre %	2.9 ± 0.05	3.1 ± 0.03	1.8 ± 0.22	1.2 ± *0.10*	1.5 ± 0.02
Carbohydrate (by difference) %	29.0 ± 0.02	28.5 ± 0.02	16.5 ± 0.14	8.1 ± 0.11	10.3± 0.50
Ascorbic Acid (mg/100 g)	18.3 ± 0.03	6.5± 0.05	12.8 ± 0.50	16.3 ± 0.14	6.5± 0.50
Reducing sugar %	1.1 ± 0.02	0.4 ± 0.05	0.5 ± 0.50	0.8 ± 0.15	0.5± 0.05
Total sugar %	2.4 ± 0.10	1.2 ± 0.02	1.5 ± 0.10	1.8 ± 0.14	1.2± 0.02
Thiamine (mg/100 g)	1.0 ± 0.10	0.4 ± 0.25	0.5 ± 0.05	1.8 ± 0.33	0.5± 0.20
Riboflavin (mg/100 g)	0.5 ± 0.05	0.2 ± 0.25	0.3 ± 0.50	0.8 ± 0.33	0.2± 0.02
Niacin (mg/100 g)	2.2 ± 0.25	1.6 ± 0.25	1.2 ± 0.14	2.7 ± 0.13	1.6± 0.10
Ca^{++} (mg/100 g)	175 ± 0.05	170± 0.33	145 ± 0.03	180 ± 0.30	170± 0.15
Fe^{++} (mg/100 g)	5.5 ± 0.02	4.3 ± 0.33	4.0 ± 0.33	4.9 ± 0.30	4.4± 0.33
$P0_4^{++}$ (mg/100 g)	280 ± 0.10	275 ± 0.33	220 ± 0.25	285 ± 0.25	276± 0.30

*All value recorded are means of replicate determination ± SE

Table 4.20: Result of Analysis of *Ogi* Samples Only

Parameters	***Ogi* Only**		
	White Maize	**Yellow Maize**	**Brown (Sorghum)**
Moisture Content %	*62.3±0.02	61.5±0.01	62.6±0.14
Protein %	3.2±0.10	3.5±0.10	3.9±0.50
Ether Extract (Fat) %	1.6±0.10	1.8±0.020	1.8±0.02
Ash %	1.3±0.05	1.3±0.02	1.5±0.01
Crude Fibre %	1.3±0.05	1.3±0.02	1.7±0.02
Carbohydrates (By Difference) %	30.3±0.02	30.6±0.02	28.5±0.02
Ascorbic Acid (mg/100g)	3.5±0.03	3.8±0.05	4.2±0.05
Reducing Sugar %	0.7±0.30	0.8±0.50	0.5±0.01
Total Sugar %	1.2±0.15	1.1±0.20	1.1±0.01
Thiamine (mg/100g)	0.4±0.50	0.7±0.02	0.3±0.02
Riboflavin (mg/100g)	0.05±0.50	0.08±0.13	0.10±0.12
Niacin (mg/100g)	0.5±0.05	0.6±0.10	0.5±0.20
Ca^{++} (mg/100g)	24±0.30	27±0.01	28±±0.01
Fe^{++} (mg/100g)	2.2±0.02	2.6±0.02	4.2±0.02
PO_4^{---} (mg/100g)	140±0.30	142±0.01	145±0.05

*All value recorded are means of replicate determinations ± SE

Table 4. 21: Result of Analysis of *Ogi* Samples Fortified With Raw and Processed Soybeans in Ratio 3:1.

Parameters	Raw Soy			Roasted Soy			Cooked Soy		
	White Maize	Yellow Maize	Brown (Sorghum)	White Maize	Yellow Maize	Brown (Sorghum)	White Maize	Yellow Maize	Brown (Sorghum)
Moisture Content %	*63.9±0.02	64.9±0.25	64.6±0.05	64.2±0.50	64.6±0.15	63.7±0.03	65.1±0.03	64.2±0.03	65.5±0.02
Protein %	11.2±0.10	11.5±0.25	13.6±0.50	11.5±0.02±0.02	11.6±0.20	13.8±0.02	10.9±0.02	11.0±0.04	11.4±0.01
Ether Extract (Fat) %	5.9±0.10	6.1±0.13	6.0±0.25	6.3±0.25	6.5±0.04	6.8±0.04	5.4±0.12	5.5±0.05	5.6±0.05
Ash %	1.8±0.14	1.8±0.12	1.7±0.05	2.0±0.12	2.1±0.20	2.2±0.02	1.4±0.02	1.5±0.03	1.5±0.05
Crude Fibre %	2.1±0.05	2.1±0.33	2.4±0.13	2.0±0.04	1.9±0.02	2.2±0.03	2.0±0.04	1.9±0.03	2.0±0.04
Carbohydrates (By Difference) %	15.1±0.02	13.6±0.30	11.7±0.14	14.0±0.03	13.3±0.03	11.3±0.04	15.2±0.13	15.9±0.13	13.0±0.20
Ascorbic Acid (mg/100g)	7.0±0.02	7.0±0.50	7.4±0.05	4.0±0.03	3.9±0.03	4.3±0.02	3.2±0.03	3.5±0.02	3.8±0.10
Reducing Sugar %	0.7±0.13	0.8±0.05	0.8±0.02	0.3±0.10	0.3±0.30	0.6±0.25	0.3±0.02	0.2±0.13	0.3±0.03
Total Sugar %	0.9±0.33	0.9±0.20	0.8±0.04	0.7±0.04	0.7±0.02	0.8±0.04	0.3±0.05	0.3±0.05	0.4±0.02
Thiamine (mg/100g)	0.8±0.02	0.9±0.02	0.7±0.12	0.4±0.04	0.4±0.13	0.7±0.50	0.6±0.04	0.7±0.02	0.5±0.02
Riboflavin (mg/100g)	0.1±0.020	0.10±0.20	0.11±0.02	0.03±0.0.12	0.03±0.03	0.15±0.30	0.09±0.02	0.09±0.13	0.10±0.05
Niacin (mg/100g)	1.5±0.30	1.6±0.01	1.9±0.12	1.3±0.20	1.3±0.13	1.5±0.04	1.1±0.03	1.1±0.02	1.3±0.20
Ca^{++} (mg/100g)	83±0.25	82±0.01	76±0.01	88±0.01	88±0.01	92±0.05	86±0.50	87±0.55	80±0.03
Fe^{++} (mg/100g)	3.5±0.50	3.6±0.14	4.4±0.10	3.2±0.55	3.2±0.13	3.8±0.04	3.1±0.50	3.2±0.02	3.2±0.03
PO_4^{---} (mg/100g)	205±0.05	208±0.20	216±0.10	226±0.55	225±0.50	228±0.04	210±0.02	212±0.55	210±0.05

*All value recorded are means of replicate determination ± SE

The table 4.22 shows the nutritional composition of the weaning food blend. There was an increase in the nutritional composition when compared with the *ogi* samples only. There was an increase in the protein content (13.8%), fat content (6.8%) an Ash content (2.2%). There was a reduction in the Carbohydrate content. There was also an increase in the vitamin content Thiamine, Riboflavin and Niacin (0.7, 0.2, 1.5 mg/100g) respectively. Analysis of variance shows a significant difference ($p=0.05$) in the sample before it was fortified with the roasted soybeans sample. The mineral content also increased significantly ($p=0.05$).

Also, when the sample was fermented together, there was a further increase in the nutritional composition. The protein content increased(17.8%),fat(12.%),Ash(3.8%),Iron(10.7mg/100g),Calcium(211.0mg/100g) and phosphate (288mg/100g). The vitamin content also increased significantly, Thiamine, Riboflavin, and Niacin (0.7, 0.2 and 1.5mg/100g respectively).

Plasmid curing affected the ability of the organism to increase the nutritional value of the food blend as there was a significant reduction ($p=0.05$) in the ability of the organism to increase the nutritional composition.

Table 4.22: Nutritional Analysis of the weaning food blend (Sorghum *Ogi*-fortified with roasted soybeans and fermented with Cured and Uncured *L.plantarum*)

Parameters	Sorghum *ogi* only	Sorghum *ogi* with roasted soybeans	Sorghum *ogi* with roasted soybeans and fermented with uncured *L.plantarum*	Sorghum *ogi* with roasted soybeans and fermented with cured *L.plantarum*
Moisture Content %	62.6±0.14	63.7±0.02	52.3±0.30	53.7±0.05
Protein %	3.9±0.50	13.8±0.15	17.8±0.30	14.0±0.14
Ether Extract (Fat) %	1.8±0.02	6.8±0.20	12.9±0.02	7.0±0.50
Ash %	1.5±0.01	2.2±0.05	3.8±0.10	2.3±0.05
Crude Fibre %	1.7±0.02	2.2±0.10	1.7±0.01	2.3±0.02
Carbohydrates (By Difference) %	28.5±0.02	11.3±0.20	11.5±0.01	20.7±0.20
Ascorbic Acid (mg/100g)	4.2±0.05	4.3±0.03	18.3±0.03	4.4±0.10
Reducing Sugar %	0.5±0.01	0.6±0.10	1.6±0.20	0.6±0.03
Total Sugar %	1.1±0.01	0.8±0.01	3.2±0.12	0.8±0.10
Thiamine (mg/100g)	0.3±0.02	0.7±0.02	1.8±0.13	0.7±0.50
Riboflavin (mg/100g)	0.10±0.12	0.2±0.03	1.0±0.50	0.2±0.05
Niacin (mg/100g)	0.5±0.20	1.5±0.50	2.7±0.03	1.6±0.13
Ca^{++} (mg/100g)	28±±0.01	92.0±0.05	211.0±0.02	92.0±0.04
Fe^{++} (mg/100g)	4.2±0.02	3.8±0.10	10.7±0.13	3.8±0.02
PO_4^{---} (mg/100g)	145±0.05	228.0±0.01	288.0±0.01	228.0±0.20

*All value recorded are means of replicate determinations ± SE

Organoleptic Attributes of the Weaning Food Blend

In a trial experiment and was used as criteria for the best weaning food composition. Sample A which consisted of roasted soy and sorghum *ogi* was preferred by the nursing mothers and was chosen as the best sample. The others were rejected but few people prefer the other samples B, C, D, therefore further work was done with sample A alone since the others samples were rejected. It was also used a criteria for selection of best weaning food for infants.

The weaning food blend were evaluated for their taste, colour, aroma, texture, flavour, odour, shelf life, acceptability and appearance based on a 5- point hedonic scale representing 'well acceptable, acceptable, fairly acceptable, moderately acceptable and reject

Table 4. 23a: Effect of curing of *L. plantarum* isolate on the organoleptic attributes of the fermented cereal blend/soybeans produced with it. It shows the effect of curing on the food blend that was prepared by adding ratio 1:3 of o*gi* and soybeans and also fermented with cured and uncured *L. plantarum* isolate. It affected the organoleptic attributes of the food blend. Samples fermented with uncured *L. plantarum* have a longer shelf life, with pleasant odour, palatable and sweet taste. The colour was creamy and was well accepted by mothers. The market survey was done among nursing mothers showed this as it is reflected in table 4.23.

Curing affected the colour of the food blend, the taste, and odour. It became irritable and unacceptable. It also has a short shelf life and the market survey by weaning mothers revealed that it was not really accepted.

Table 4. 23b: Effect of curing of *L. plantarum* isolate on the organoleptic attributes of the fermented cereal blend/soybeans produced with it. Table 22b Shows the effect of plasmid curing on the organoleptic attributes of the food blend that was fermented either with cured or uncured *L. plantarum* isolates. Samples fermented with uncured isolates were well accepted while the ones fermented with cured isolates were rejected.

Table 4.23c below shows the Street Survey among nursing Mothers on *Ogi* Preparation and Weaning Food Fortification and Supplementation. It could be observed that very few mothers have knowledge about the complete fermentation of *ogi* and the work of

L. plantarum in weaning foods. Various superstitious beliefs also abound for the presence of gas production, bloating and flatulence in young infants.

Table 4. 23a: Effect of curing of *L. plantarum* isolate on the Organoleptic attributes of the fermented cereal blend/soybeans produced with it

Organoleptic Attributes	Roasted Soybeans +Sorghum *Ogi* (A)	Roasted Soybeans + Dried Sorghum *Ogi* (B)	Roasted Soybeans + Sorghum *Ogi* fermented with Uncured L.*plantarum* (C)	Roasted Soybeans + Sorghum *Ogi* fermented with cured L.*plantarum* (D)
Taste	Sweet	Sweet	Sweet	Sour
Colour	Creamy	Creamy	Creamy	Grey
Aroma	Acceptable	Fairly acceptable	Moderately acceptable	Irritable
Texture	Acceptable	Fairly acceptable	Moderately acceptable	Not acceptable
Flavour	Acceptable	Fairly acceptable	Moderately acceptable	Not acceptable
Odour	Acceptable	Fairly acceptable	Moderately acceptable	Not acceptable
Shelf life	2 months	2½ months	2 months	3 days
Acceptability	Well accepted	Fairly acceptable	Moderately acceptable	Rejected
Appearance	Well accepted	Fairly acceptable	Moderately acceptable	Rejected

Table4. 23b: Survey Carried Out Among Nursing Mothers on the Organoleptic Attributes of the Weaning Food Blend

Organoleptic Attributes	A	B	C	D
Taste	5^a	3^b	2^c	1^d
Colour	5^a	3^b	2^c	1^d
Aroma	5^a	3^b	2^c	1^d
Texture	5^a	3^b	2^c	1^d
Flavour	5^a	3^b	2^c	1^d
Odour	5^a	3^b	2^c	1^d
Shelf life	5^a	3^b	2^c	1^d
Acceptability	5^a	3^b	2^c	1^d
Appearance	5^a	3^b	2^c	1^d

Key: **Sample A**=Roasted Soybeans+Sorghum *Ogi*

Sample B= Roasted Soybeans + Dried Sorghum *Ogi*

Sample C=Roasted Soybeans + Sorghum *Ogi* and Fermented Uncured L.*plantarum*

Sample D =Roasted Soybeans + Sorghum *Ogi* and Fermented cured L.*plantarum*

Table 4.23c: Street Survey Among Nursing Mothers on *"Ogi"* Preparation and Weaning Food Fortification and Supplementation

Sampling Population: 300

Parameter Required	%
% that use *'Ogi'* as weaning food	21%
% that soak for 2 days or buy the item (incomplete fermentation)	5%
% that soak for 3 days (incomplete fermentation)	5%
% that soak for 5 days (complete fermentation)	10%
Women that understand the meaning and cause of bloating, gas production or flatulence in children	5%
Women with superstitious beliefs about bloating and gas production	40%
% that claim that the product is cheap and easy to prepare	80%
Those that understand the importance of lactic acid bacteria	2%
Those that use canned foods, indomie and others	10%
Those that use food supplement like honey, soybeans	50%
Those that use sweeteners and other flavours	40%
Those that believe that complete fermentation reduces *ogi* swelling	65%

Sites Chosen: Bodija Market and Ibadan North Local Government, Ibadan

Table 4.24 shows the effect of plasmid curing on the cellular morphology of the isolates. Curing affected the shape, size, colour, surface, and elevation etc of the isolates.

The table shows the effect of plasmid curing on the cellular morphology and characteristics of the isolates. It affected these negatively. The shape became irregular, colour became grey. It became flat, not entire and not spreading and rough as against the circular, creamy, raised, entire and smooth in the uncured sample. Curing had a negative effect on the cellular morphology of the isolate.

Table 4. 24: Effect of Curing on the Cellular Morphology of selected 9 *L.plantarum* Isolates

Cellular Morphology	**Uncured *L.plantarum***	**Cured *L.plantarum***
Shape	Circular	Irregular
Size	Small	Big
Colour	Creamy	Grey
Elevation	Slightly raised	Flat
Margin	Entire	Not Entire
Edges	Smooth	Rough
Surface	Wet	Wet/spreading
Pigmentation	Nil	Nil

Figure 4.23 shows a trend in the fermentation pattern when the uncured and cured *L. plantarum* was to ferment the cooked soybeans sample. There was a significant increase (p=0.05) in the reducing sugar production (mg/ml) with the uncured *L. plantarum* sample from 0 hrs to 96 hrs, there was a drop at 120 hrs. It increases from (0.58 mg/ml at o hr to (0.98 mg/ml at 72 hrs and the peak at 96 hrs (1.1 mg/ml) but it reduced to (0.84 mg/ml at 120 hrs.

Using cured *L. plantarum* isolate for fermentation, a significant increase (p=0.05) in the reducing sugar was not observed until the 72 hrs of fermentation, 0 hrs to 48 hrs were almost the same (0.52, 0.54 mg/ml, at 72 hrs the reducing sugar content was (0.62 mg/ml) and a reduction at 120 hrs and 96 hrs with (0.52 mg/ml and (0.54 mg/ml) respectively. There was thus a significant difference (p=0.05) between cured and uncured *L. plantarum.*

Figure 4.24 shows the reducing sugar (mg/ml) production by cured and uncured *L. plantarum* isolate in the same sample when the uncured isolate was used, there was a gradual increase in the reducing sugar produced from 0 hrs to 96 hrs being the peak, (0.52 mg/ml) and 0 hr (0.62 mg/ml) 48 hrs and (0.96 mg/ml) at 96 hrs. There was a significant reduction (p=0.05) at 120 hrs to (0.85 mg/ml).

Using cured *L. plantarum*, the reducing sugar did not increase at all as the fermentation progressed; it remained at 0.5 mg/ml from 0 hr to 0.52 at 48 hrs and 0.52 at 96 hrs and 120 hrs respectively. There was a significant difference (p=0.05) between cured and uncured *L. plantarum*

Figure 4.25 shows the reducing sugar production (mg/ml) by the cured and uncured *L. plantarum* isolate in the roasted sample. There was an increase in the reducing sugar from 0 hr to 72 hrs respectively, from 0.62 mg/ml to 0.72 mg/ml and 1.0 mg/ml. The peak was observed at 96 hrs 1.8 mg/ml and a reduction to 0.98 at 120 hrs.

Using cured *L. plantarum*, there was no significant increase (p=0.05) from 0 to 42 hrs (0.52 mg/ml), a slight increase was observed at 72 hrs (0.57 mg/ml), there was a reduction at 96 hrs to (0.52 mg/ml), and 0.5 mg/ml at 120 hrs. There was a significant difference (p=0.05) between cured and uncured *L. plantarum.* Analysis of variance result shows a significant difference (p=0.05) in the various soybeans sample, the highest was observed in roasted (1.8 mg/ml), this was followed by cooked (1.1 mg/ml) and the least was observed in raw (0.96 mg/ml), these being the peak in all the samples. There was also a significant difference (p=0.05) in the various time intervals as significant changes took place in the cooked and roasted samples but this was not observed in the raw samples.

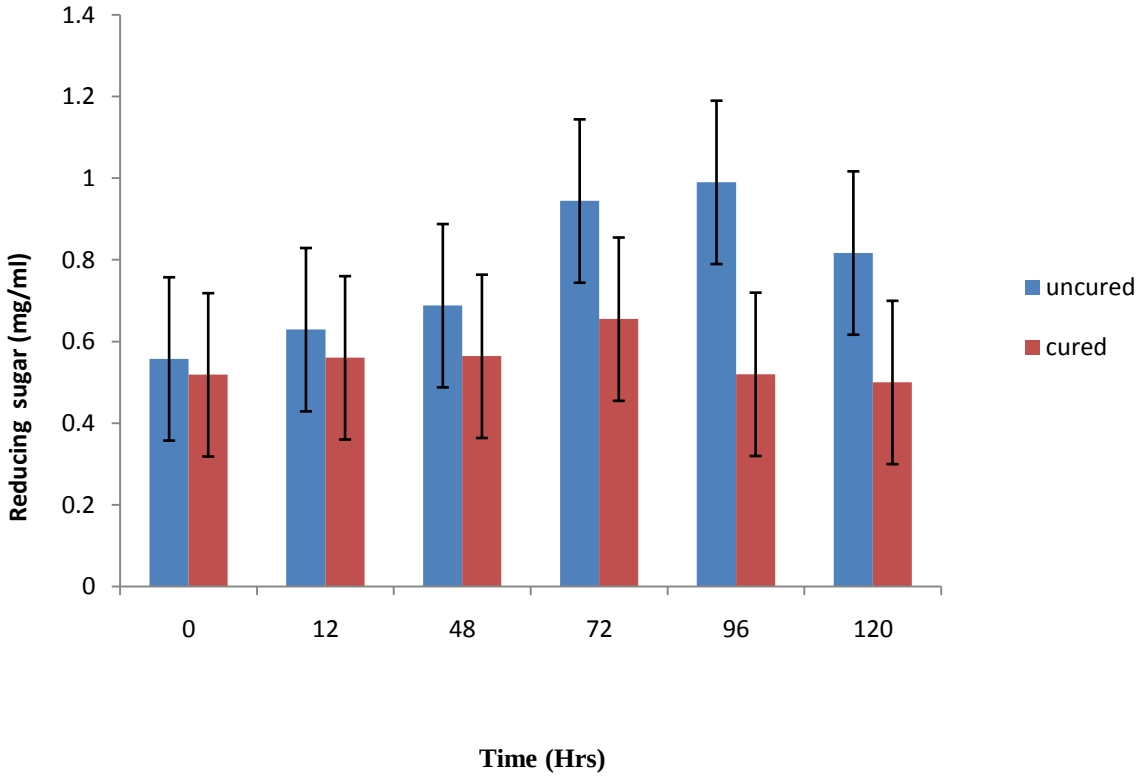

Fig. 4.23: Timed profile of Reducung Sugar production by *L. plantarum* isolate LV1, LV2 and LV3 during fermentation with cooked Soybean

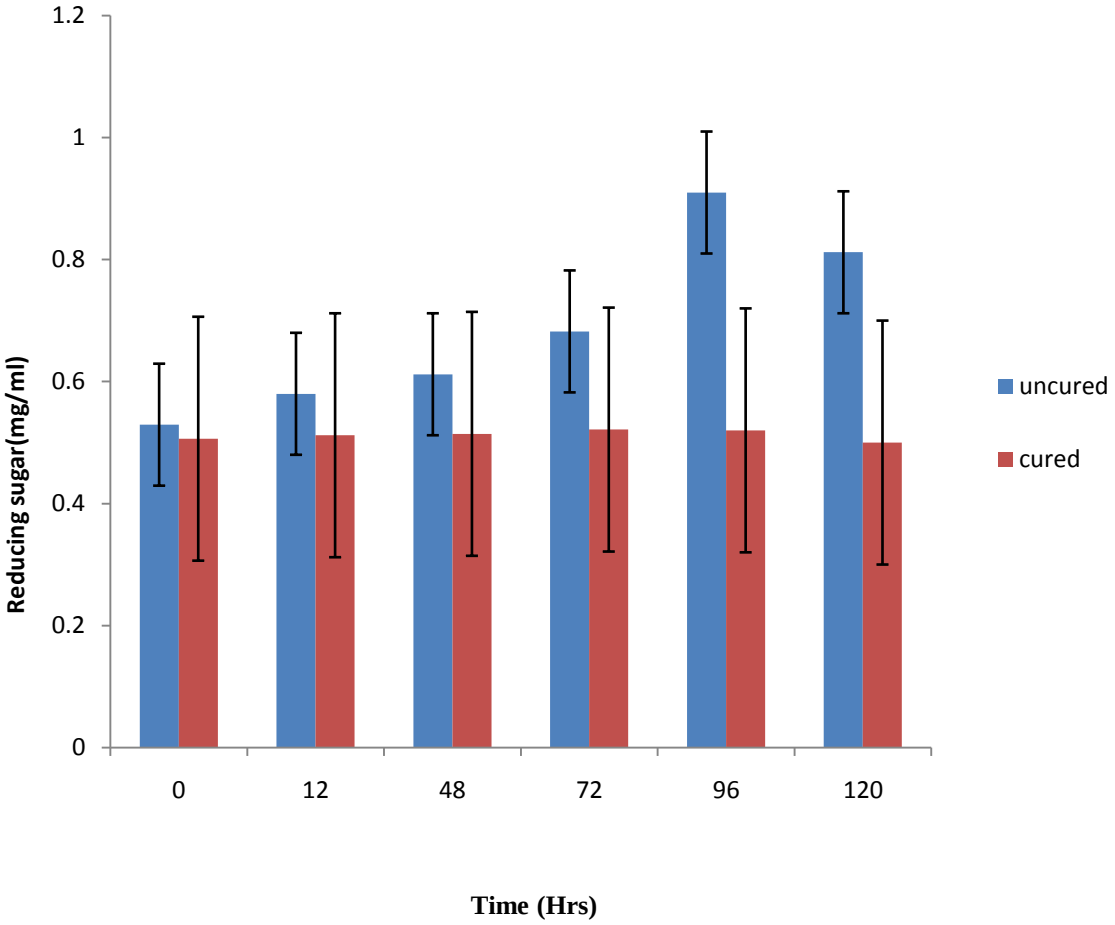

Fig. 4.24: Timed profile of Reducing Sugar Production by *L. plantarum* Isolate LV1, LV2 and LV3 During Fermentation from Raw Soybeans

Values are means of replicate determinations, significant level (P<0.05)

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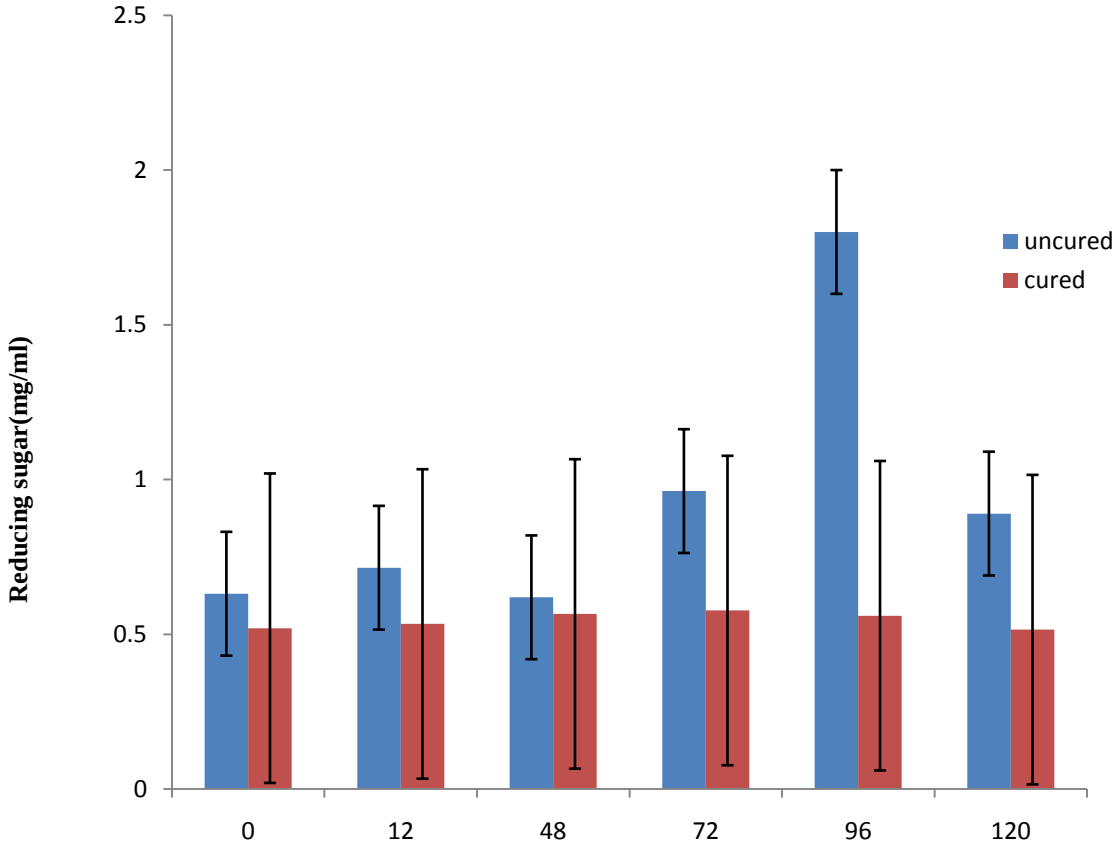

Fig. 4.25: Timed profile of Reducing Sugar Production by *L. plantarum* Isolate LV1, LV2 and LV3 During Fermentation from roasted Soybeans

Values are means of replicate determinations, significant level (P<0.05)

Figure 4.26 shows the comparative oligosaccharide content (mg/100mg)[1] from cooked soybeans fermentation by uncured and cured *L. plantarum* isolate. Cooking has reduced the oligosaccharide content of the soybeans before fermentation compared to what was found in raw (3.6) to (1.7). With cooked soybeans, there was a reduction in the oligosaccharide content from o hr to 120 hrs. No significant reduction (p=0.05) was observed between 0-24 hrs, but at 48 hrs of fermentation, there was a reduction from 1.2 to 1.0 at 48 hrs, 0.9, 0.8 and 0.6 at 72, 96 and 120 hrs respectively. Using cured isolate, the oligosaccharide content did not reduce so much, there was no significant difference (p=0.05) in the reduction process from 24hrs to 120 hrs(3.6 t0 3.5).

Figure 4.27 shows the comparative oligosaccharide content (mg/100mg)[1] of raw soybeans subjected to fermentation by cured and uncured *L. plantarum*. The oligosaccharide content of the raw sample at 0 hr was 3.6, using the uncured isolate, there reduction was rather slow. No observable change was noticed until the 48 hrs (2.6). The oligosaccharide content reduced from (1.7) to (1.2) at 96 hrs and at 120 hrs to (0.9).

Using the cured *L. plantarum*, the oligosaccharide content was much and did not reduce. There was no significant difference observed. The oligosaccharide content remained the same from 0 hr to72hrs and 120 hrs at (3.5 to 3.5 and 3.4) respectively.

Figure 4.28 shows the comparative oligosaccharide content (mg/100mg)[1] of the roasted soybeans subjected to fermentation by cured and uncured *L. plantarum*. Roasting has reduced the oligosaccharide content a lot before fermentation from (3.6) in raw to (1.4). The highest oligosaccharide content observed was (1.4), it reduced from (1.4) to (1.1) at 24hrs hrs, then to (0.9),(0.8),(0.7) and (0.6) at 48, 96 and 120 hrs respectively. There was a significance reduction (p=0.05) in the oligosaccharide content from 0 hr-120 hrs when the uncured sample was used. The pre-treatment method had a lot of significant difference (p=0.05) on the oligosaccharide content as it was possible for fermentation to start immediately at 24hrs.

With the cured sample, the oligosaccharide content did not reduce significantly(p=0.05), the reduction was rather very slow, no observable changes from 0-48 hrs, the noticeable change was observed from 48 hrs to 72 hrs (1.3),72 to 96 hrs (1.3) and (1.2) at120hrs respectively. Curing affected the reduction of oligosaccharide content during fermentation by *L. plantarum* isolate. The analysis of

variance result shows a significant difference (p=0.05) between the Raw, cooked and roasted soybeans in the reduction of oligosaccharide content, while it was the highest in raw sample (3.6), it was followed by cooked (1.7) and the least was observed in roasted (1.4).

There was a significant difference (p=0.05) in the time interval from 0 – 120 hr, also there was a significant difference (p=0.05) in the oligosaccharide content between the cured and uncured *L. plantarum* isolates that was used for fermentation.

Figures 23, 24 and 25 show the effect of fermentation on the reducing sugar (mg/ml) content of the pre-treated soybeans. It could be observed that there was a reduction in the oligosaccharide content from 0hrs to 96 hrs when the uncured *L.plantarum* was used for fermentation. When the cured isolate was used, there was no reduction in the oligosaccharide content at all, the cured isolate could not break down the oligosaccharide content ,it remain the same as the fermentation progressed.

The reducing sugar content of the food blend increased from 0hrs to 96hrs when uncured *L.plantarum* was used for fermentation. The oligosaccharides in the samples were broken down by the organism into reducing sugar. At 120 hrs, the reducing sugars reduced again. But when the cured isolate was used, there was no increase in the reducing sugar content at all, the ones that was observed was very small. This is because plasmid curing affected the ability of the organisms to break down oligosaccharide content of the samples into reducing sugars.

Figures 26, 27 and 28 show the oligosaccharide content (mg/100mg)[1] of the samples as the fermentation progresses. *L.plantarum* was able to reduce the oligosaccharide content of the samples, even though the different pre-treatment methods have reduced the oligosaccharide content to an extent (Table 4.18), fermentation with *L. plantrum* was able to break it down further, and there was reduction in the oligosaccharide content from 0 hrs to 120 hrs as observed in the samples.

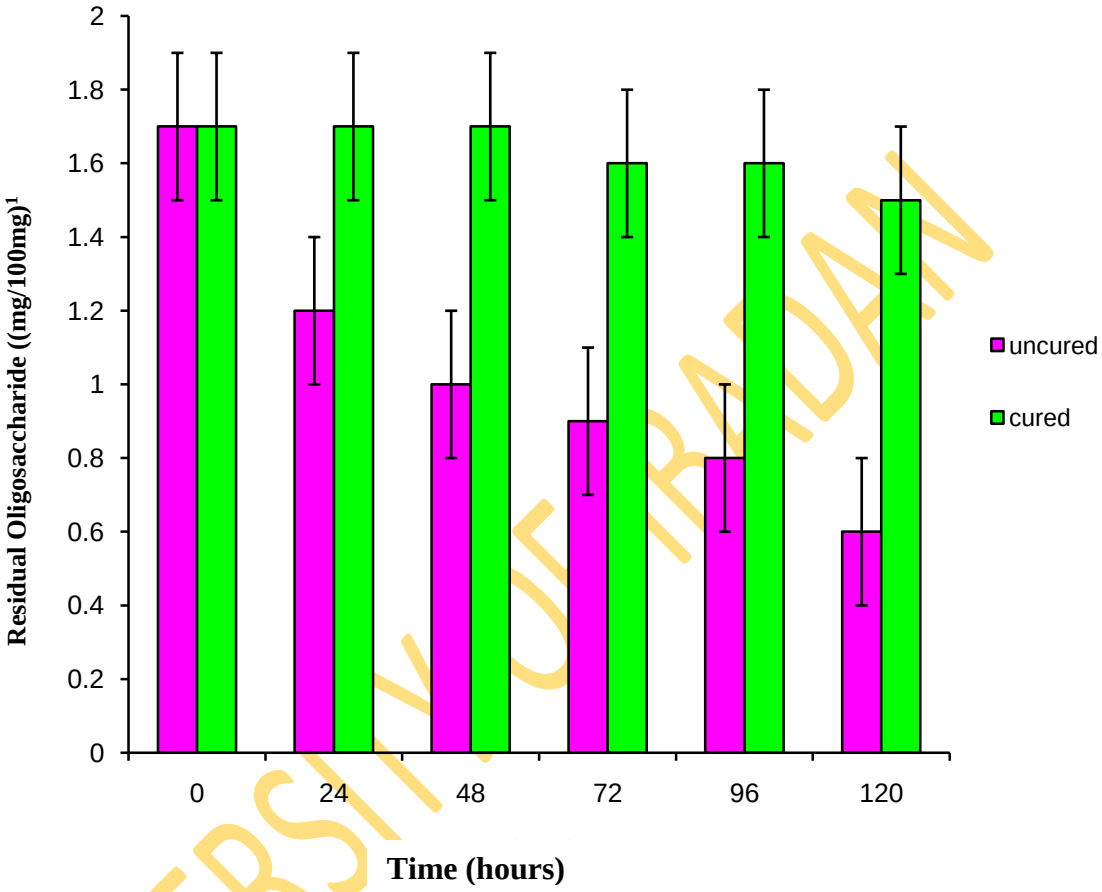

Figure 4.26: Residual Oligosaccharide Content From Cooked Soybeans During Fermentation

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Values are means of replicate determinations shows significance in cured and uncured ($P<0.05$)

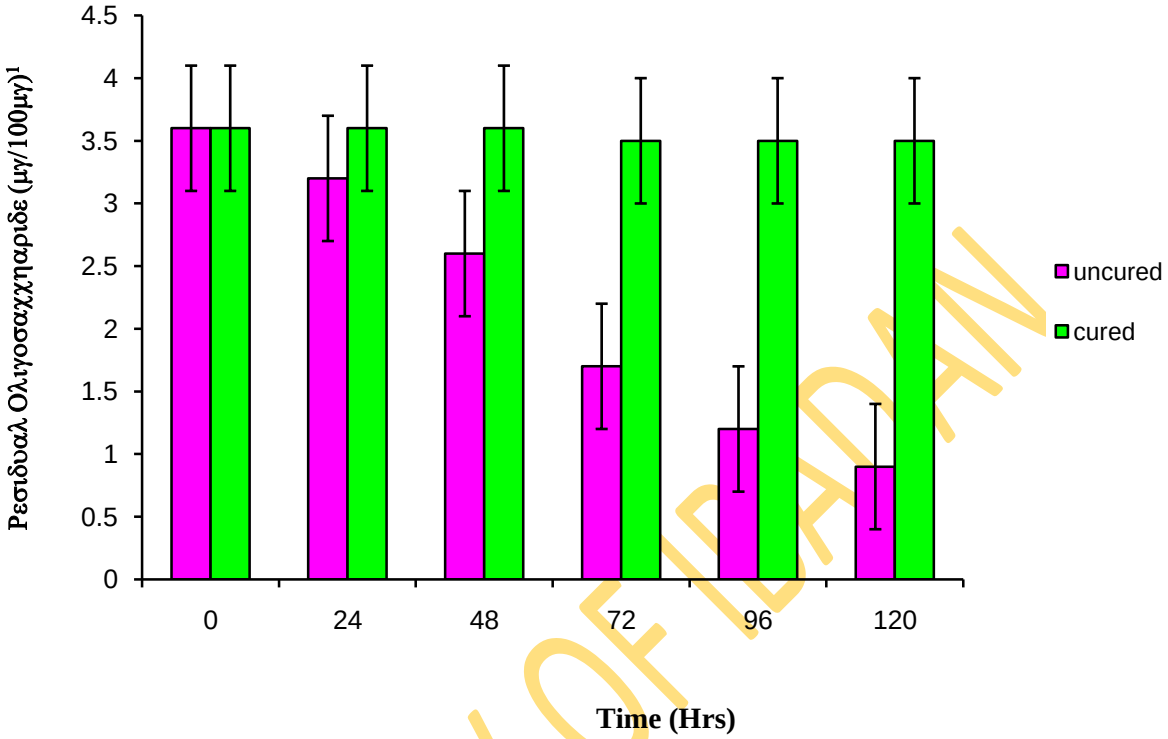

Figure 4.27: Residual Oligosaccahride Content From Raw Soyabeans During Fermentation by *L. plantarum* Isolate LV1, LV2 and LV3

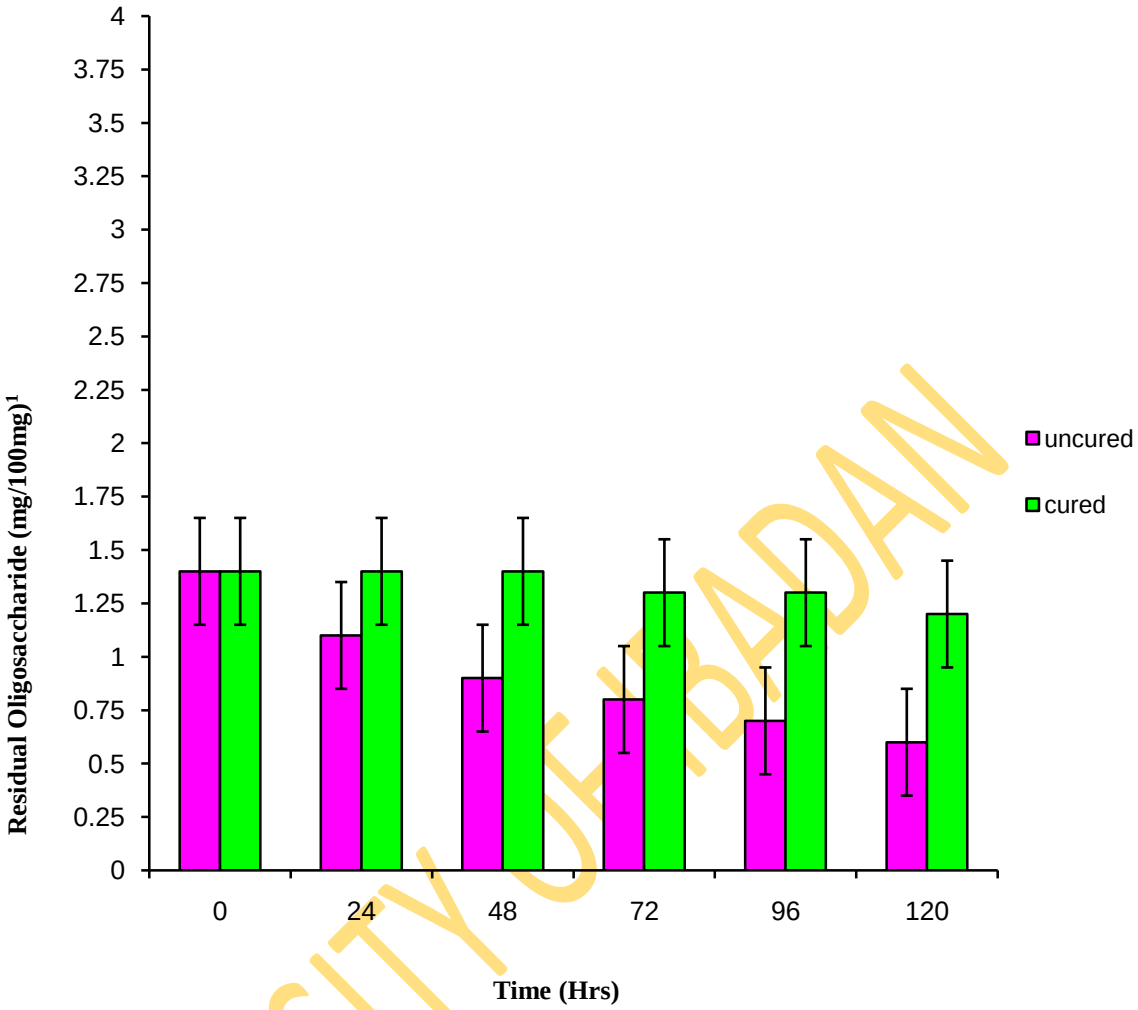

Figure 4.28: Residual Oligosaccahride Content From Roasted Soyabeans During Fermentation by *L. plantarum* Isolate LV1, LV2 and LV3

Figure 4.29 Shows the oligosaccharide profile content (mg/100g)[1] of Raw soybeans during fermentation by the selected *L. plantarum* isolate. It will be observed that there was a decrease in the profile of Raffinose, stachyose and verbascose as the fermentation progressed from 0hr – 120hrs. Stachyose was the highest in all the sugars (2.838) at 0 hr; this was followed by Raffinose (0.324) while verbascose was the least (0.256).

A 28% reduction in the oligosaccharide profile was observed in Raffinose, in stachyose, from 0hr – 96hrs. In stachyose, it was a 29% reduction, while in verbascose; it was a 37% reduction. Analysis of variance shows a significant difference (p=0.05) in all the sugars and the time interval.

Figure 4.30 shows the oligosaccharide profile content of cooked soybeans (mg /100g)[1] during fermentation by the selected *L. plantarum* isolate.

It was observed that there was a decrease in the sugar profile from 0hr – 120hrs respectively. Stachyose was the highest (1.126), this was followed by Raffinose (0.176) and verbascose was the least (0.150). There was a 72% reduction in stachyose from 0-120hrs, 58% reduction in raffinose from 0-120hrs and 41% reduction in verbascose from 0-96hrs as the fermentation progressed. Analysis of variance shows a significant difference (p=0.05) in all the sugars and the various time intervals.

Figure 4.31 shows the oligosaccharide profile content of Roasted soybeans (mg/100g)[1], during fermentation by the selected *L. plantarum* isolate.

It was observed that there was a decrease in the sugar profile from 0-120hrs respectively. Stachyose was the highest (1.036), This was followed by Raffinose (0.152) while verbascose was the vast (0.124). There was however a 76% reduction is stachyose, 68% in Raffinose and 71% in verbascose during fermentation.

Analysis of variance shows a significant difference (p=0.05) between the 3 sugars and the time interval.

The various pre-treatment methods have however reduced the oligosaccharide content in soybeans to an extent in (mg/100)[1] when compared to the raw samples of soybeans. However, there was a significant difference (p=0.05) in the sugar profile between the raw, roasted and cooked. The percentage of the Raffinose Oligosaccharide Family was highest in the raw (3.212), this was followed by cooked (2.124) and the least was observed in roasted (1.112). This same trend was observed along the different time intervals.

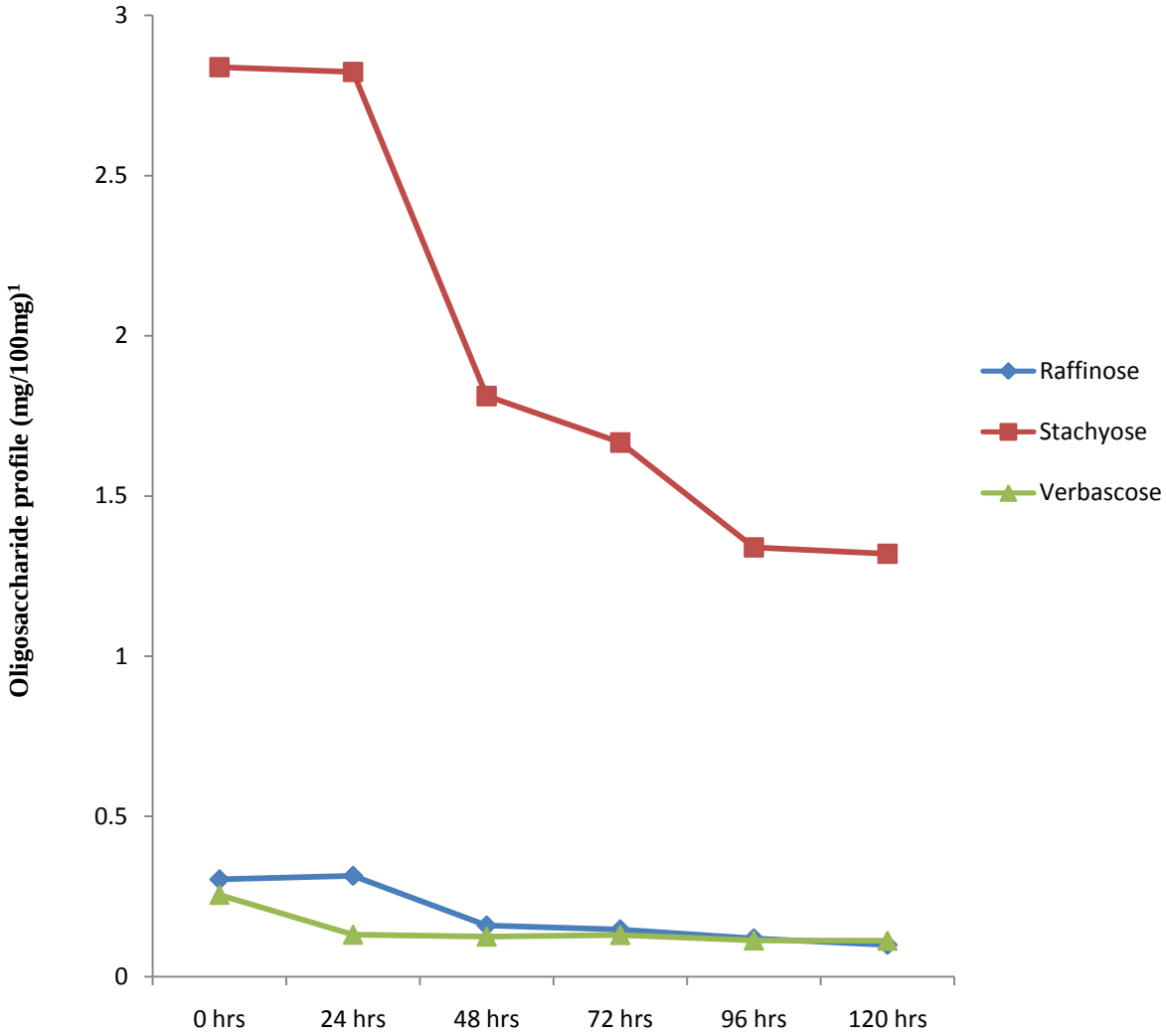

Fig. 4.29: Oligosaccharide production profile in Raw soybeans during fermentation with the selected *L. plantarum* isolate(LV1)

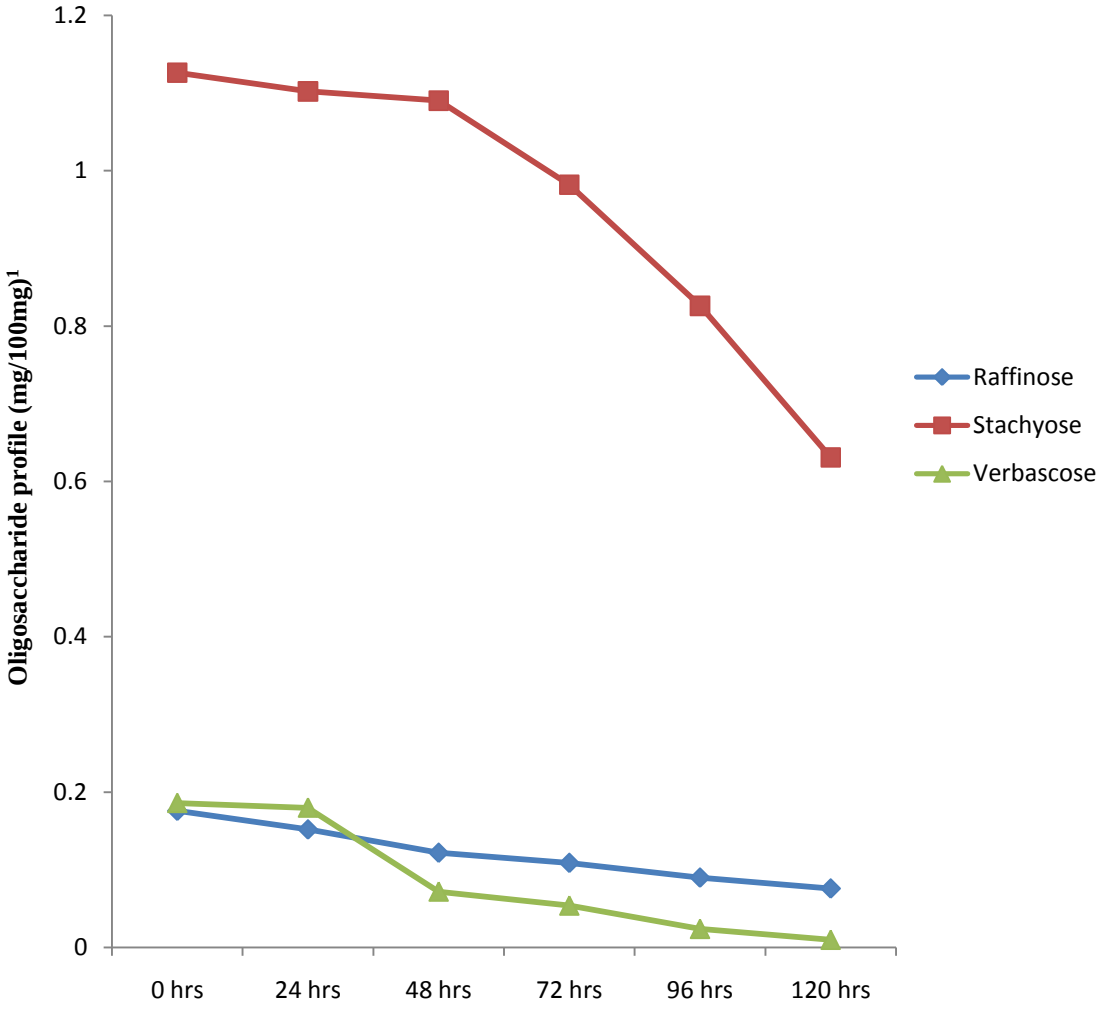

Fig. 4.30: Oligosaccharide production profile in cooked soybeans during fermentation with the selected *L. plantarum* isolate(LV1)

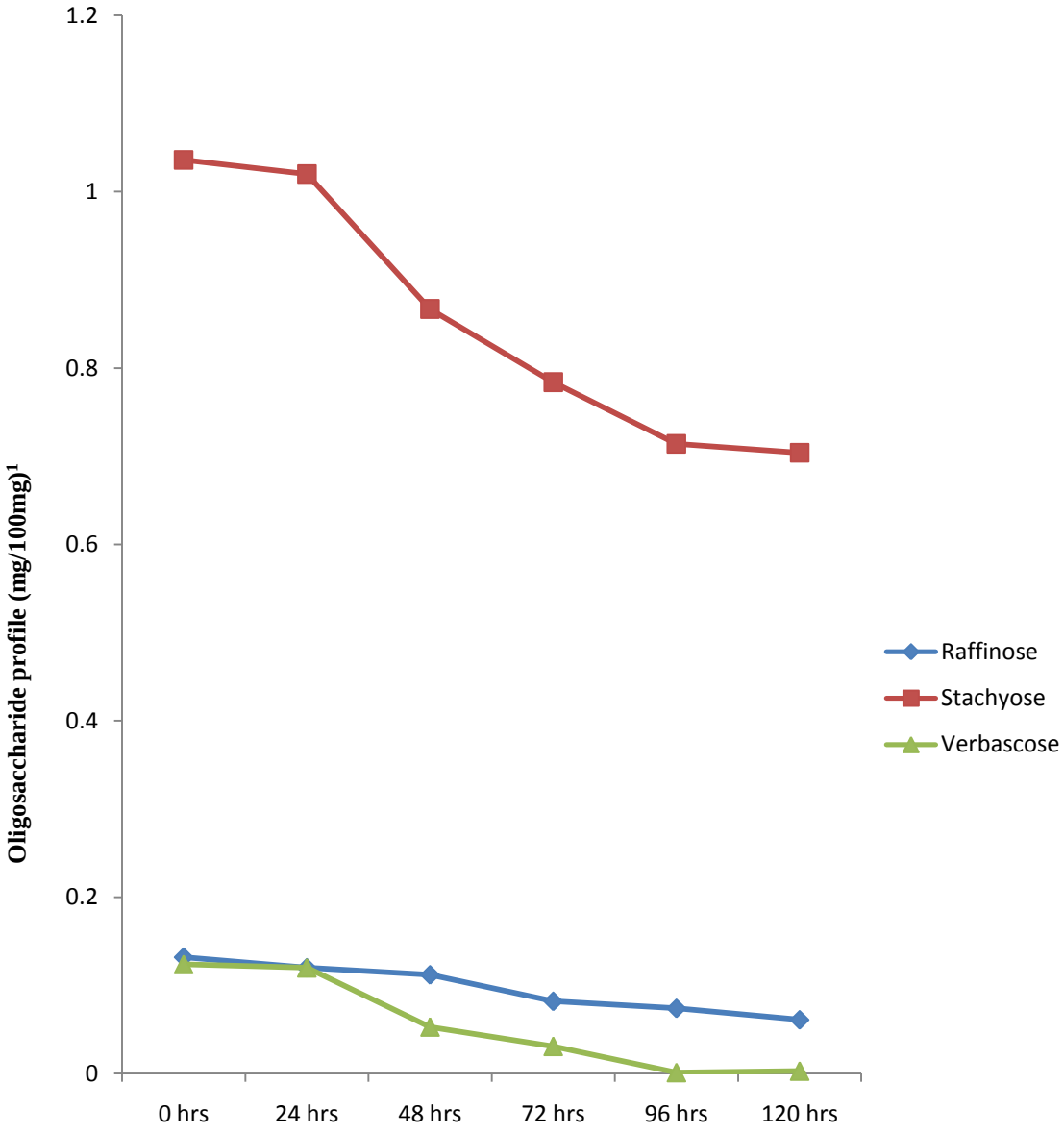

Fig. 4.31: Oligosaccharide production profile in Roasted soybeans during fermentation with the selected *L. plantarum* isolate(LV1)

Figure 4:32 Shows the reducing sugar profile (mg/ml) of Raw Soybeans during fermentation by the selected the *L. plantarum* isolate. There was an increase in the sugars produced (Glucose, fructose and sucrose) as the fermentation time progressed from 0hr to 96hrs. (0.095-1.165) respectively. There was however a drop at 120hrs. This means that there was an increase of 61% in glucose, 53% in fructose and 32% in sucrose (reducing sugars) produced during fermentation. Also, there was a significant difference (p=0.05) in the sugars produced different time interval.

Figure 4:33 show the reducing sugar profile (mg/ml) of cooked soybeans during fermentation by the selected *L. plantarum* isolate. There was an increase in the sugars produced (glucose, fructose and sucrose) as the fermentation time progressed (0-96hrs). A decrease was also observed at 120hrs. This means that there was an increase of 78% in glucose,64 % in fructose and 44% in sucrose (reducing sugars) profile during fermentation. Also, there was a significant difference (p=0.05) in the sugar produced at different time interval.

Figure 4:34 shows the reducing sugar profile (mg/ml) of Roasted soybeans during fermentation by the selected *L. plantarum* isolate. There was an increase in the sugars produced as the fermentation time increased from 0-96hrs. A decrease was however seen at 120hrs. This means that there was a significant difference (p=0.05) in the reducing sugar profile during fermentation.

Analysis of variance shows a significant difference a significant difference (p=0.05) in the reducing sugars produced in the Raw, Cooked and Roasted Soybeans. The sugars produced in the roasted samples were the highest (1.265). This was followed by cooked (1.042) while the raw sample was the least (0.670).

There was a significant decrease in the sugar produced from 96 hrs to 120hrs. (1.265 to 0.801) in Roasted, (1.042 to 0.760) in cooked and (0.670 to 0.614) in the raw samples.

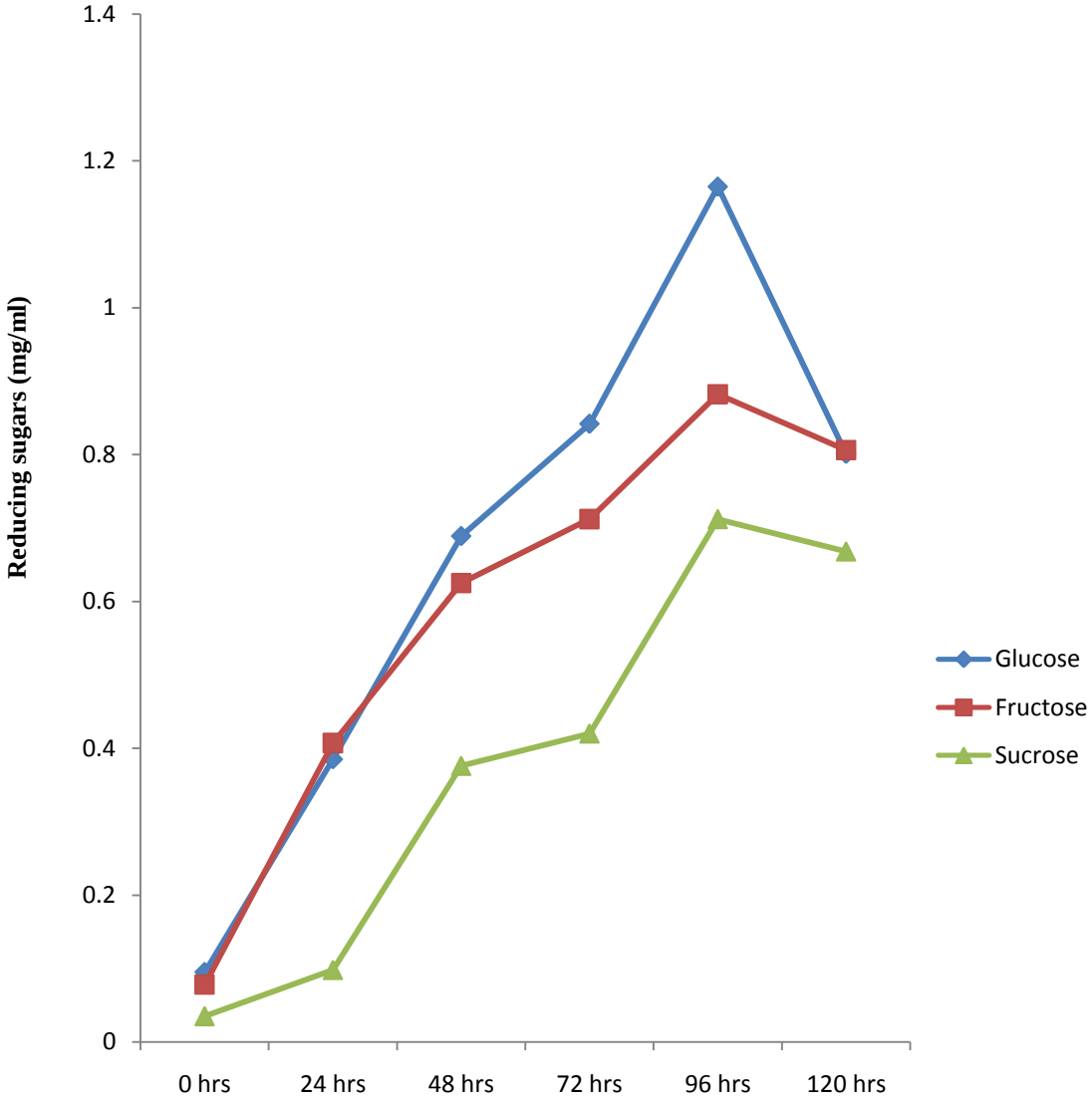

Fig. 4.32: Reducing sugars profile of raw soybeans produced during fermentation with selected *L. plantarum isolate(LV1)*

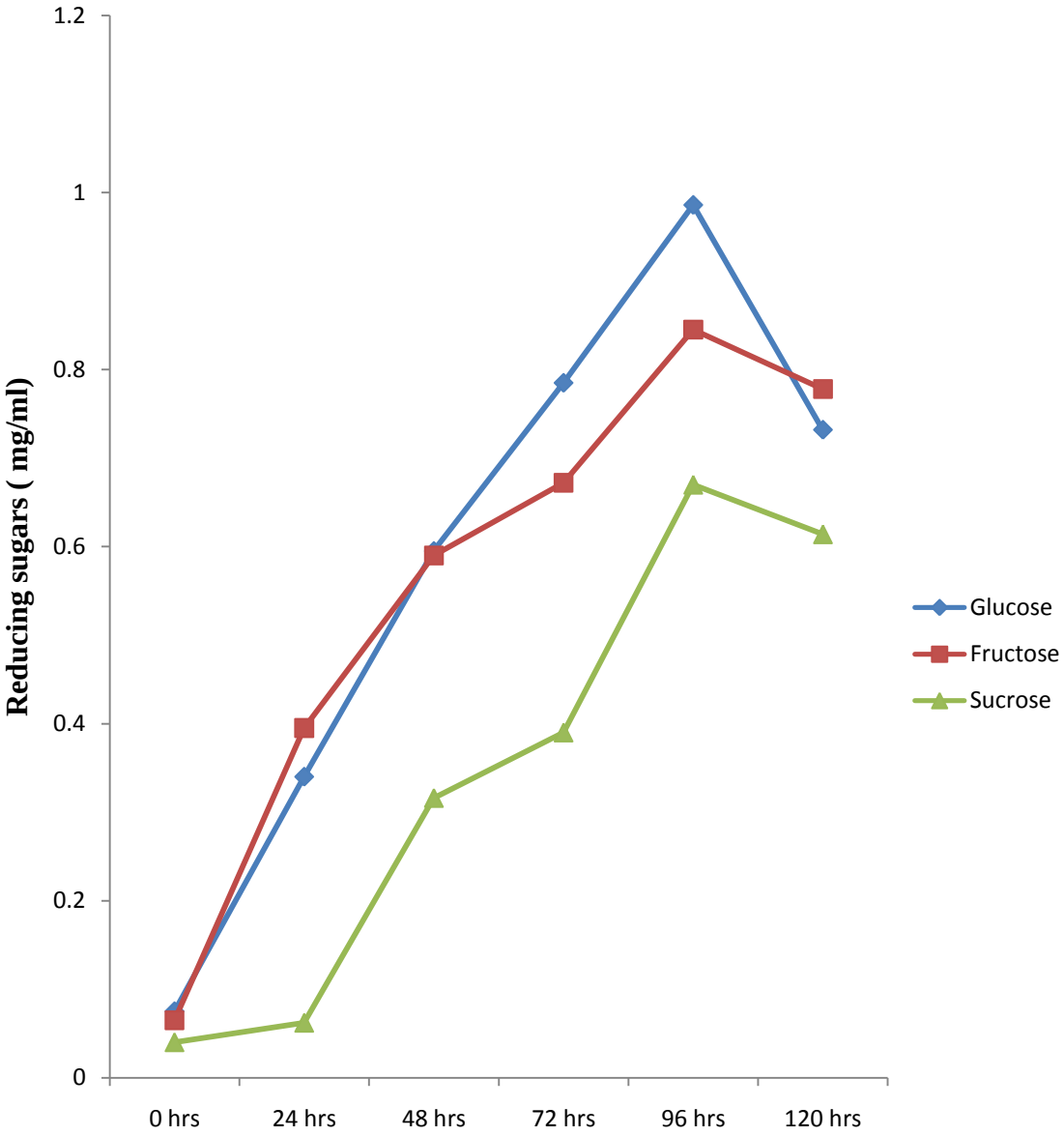

Fig. 4.33: Reducing sugars profile of cooked soybeans produced during fermentation with selected *L. plantarum* isolate (LV1)

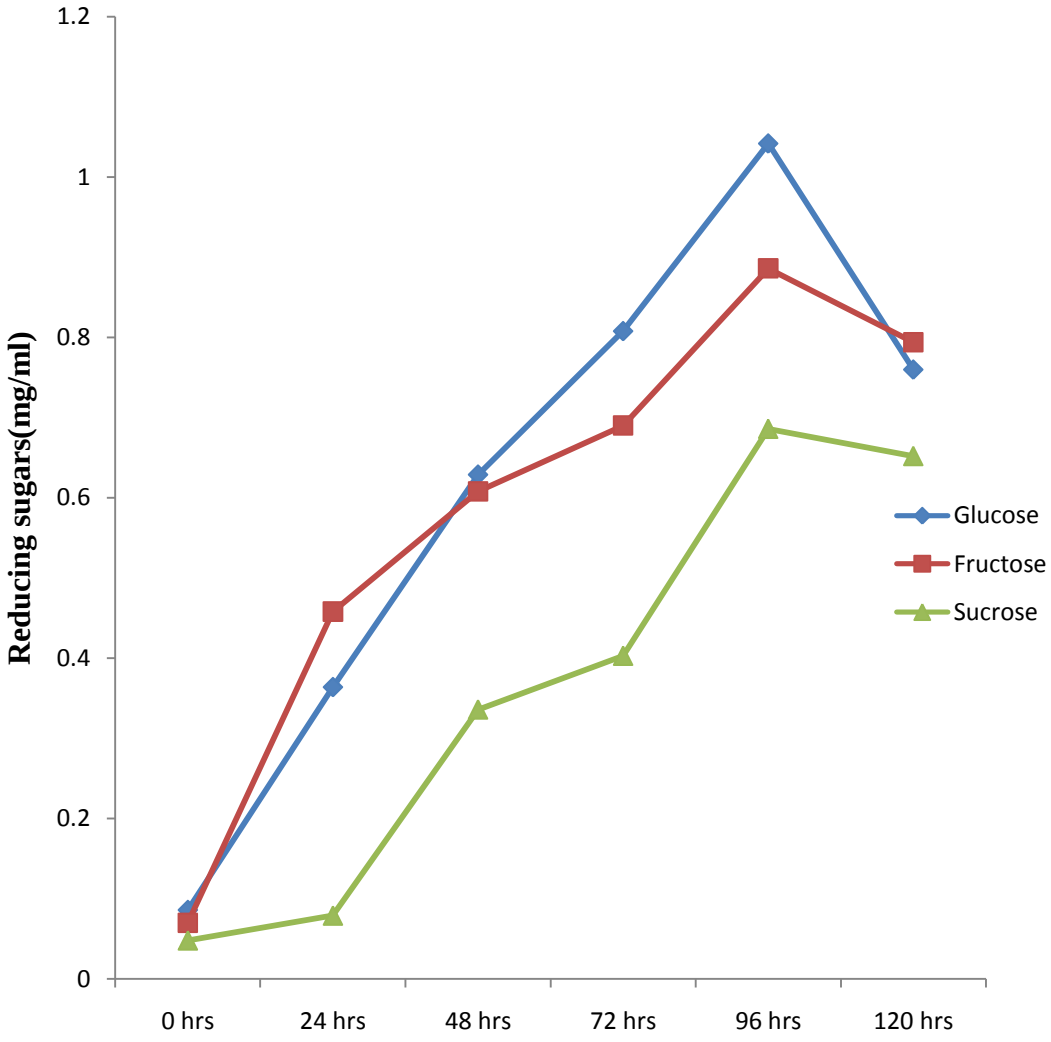

Fig. 4.34: Reducing sugars profile of roasted soybeans produced during fermentation with selected *L. plantarum* isolate(LV1)

CHAPTER FIVE

5.0 DISCUSSION

During the last decades, due to the beneficial impact of microorganisms used as probiotics on human and animal health, progressive attention has been focused on biological and molecular characterization and improvement of such microbes. Most work that have been done in the past have revealed that LAB are a major microbial group having beneficial effects on the health status of host organisms and man (Vassu *et al.*, 2002;Amankwah *et al.*, 2012).

The beneficial effect of probiotics are based on many known mechanisms out of which the most important ones are: inhibition of intestinal pathogenic bacteria by the production of organic acids and by pH reduction, prevention of pathogens' adherence to the intestinal mucosa, production of bacteriocins and other antimicrobial agents, reduction of raffinose-oligosaccharides and anti-nutritional factors such as cyanide and tannins in food. Others include increase of food assimilation and detoxification processes, improvement of nutritional and organoleptic factors of food like flavour, taste and aroma etc (De Vuyst and Vandamme, 1994; Gomez and Malcata, 1998; Gray *et al.*, 2012).

The Raffinose oligosaccharide consists of the trisaccharide Raffinose, tetrasaccharide stachyose and pentasaccharide verbascose. The three sugars are complex oligosaccharide which cannot be easily broken down into simple sugars in the intestinal tract of man resulting in bloating, flatulence and abdominal pain in man. The enzyme alpha galactosidase is needed to break it down into its simple forms. Raffinose oligosaccharide can be hydrolysed and broken down into its simple sugars by the metabolic activities of *L. plantarum*. Complete hydrolysis of raffinose gives 1 mole each of D-galactose, D-glucose, and D-fructose. In structure, it comprises mellibiose and sucrose with the central D-glucose in common (Storey *et al.*, 1998; Gray *et al.*, 2012).

The complete hydrolysis of raffinose is able to solve the problem that is usually encountered by the consumption of food that contains raffinose-oligosaccharide i.e.bloating, abdominal pain and flatulence. Thus, the enzymatic process in which *L. plantarum* has been implicated in the breakdown of raffinose into simple sugars because it produces alpha and beta galactosidase enzyme in abundance has been studied (Vitobello *et al.*, 1992).

In this study, Lactic acid bacteria were isolated from three major sources and the organisms were subjected to various physiological processes in order to select the ones that were suitable for the purpose of breaking down the raffinose family of oligosaccharide (RFO) in food. The various species of lactic acid bacteria that were isolated from the samples were 120 in number, and they were identified as *L. plantarum* (43), *L. fermentum* (15), *L. acidophilus* (19), *L. brevis* (08), *L. casei* (07), *L. pentosus* (09), *L. cellobiosus* (08), *L. jensenii* (06) and *L. reuterii* (05).The results obtained are in accordance with the observation that LAB are part of the flora that dominate cereal fermentations which is consistent with the findings of Olsen *et al*.(1995) and Olukoya *et al*.(1993) on isolation of LAB. Their identities were also confirmed by molecular methods.

Olukoya *et al.* (1993) have reported the dominance of lactic acid bacteria in fermentation of cereals. The result obtained from this research is in agreement with the works that have been reported by some authors before and this is in agreement with such works including Oyewole and Odunfa (1990), Onilude *et al.* (1993) and Olsen *et al.* (1995) that implicated these organisms as being responsible for the fermentation of naturally- fermenting cereals which are a major part of the foods commonly consumed in West Africa. This is because of their ability to tolerate any medium or substrate with a lower pH range. This aspect partially enables LAB to outcompete other bacterial groups in a natural fermentation, as they can withstand the increased acidity from organic acid production e.g. lactic acid and acetic acid (Wakil and Onilude, 2009).

The work of Hammes and Tichazek (1994) also stated the potential importance of LAB especially *L. plantarum* in the production of fermented foods. These constitute one quarter of the diet we consume and are characterized by a safe history, certain beneficial health effects, and an extended shelf life when compared with raw materials. The various fermenting substrates are habitats for specific LAB that differs in their metabolic potential. Hammes and Tichazek (1994) also stated the health effects exerted by LAB as follows. First, is the production of lactic acid and minor amounts of acetic and formic acid. These cause a drop in pH and thereby growth inhibition of food spoilage or poisoning bacteria; killing of certain pathogens; detoxification by degradation of noxious/complex compounds of plant origin (usually in combination with plant-derived enzymatic activities). Secondly, they reported the production of antimicrobial compounds (e.g. bacteriocins,

Hydrogen peroxide, diacetyl and various organic acids) and probiotic effects as live organisms in food. The wholesomeness of LAB can also be extended to fields outside human nutrition, as they may act as probiotics in animal production or as plant protectives in agriculture thus contributing to healthy raw materials for food production according to Hammes and Tichazek (1994).

Furthermore, fermentation is one of the ways of preserving food substances even in the olden days. This is because of the ability of LAB to prolong the shelf life of food substances (Oyewole and Odunfa, 1990). LAB act as probiotics in food preparation and preservation and as such are able to lower the pH of the food in which they are found making it unsuitable for spoilage microorganisms to survive therein and cause spoilage. Thus, they able to prolong the shelf life of such food substances (Oyewole and Odunfa, 1990).

In the past few years, the phenotypic identification and characterization of organisms has shown some limitations. The basis of identification on their utilisation of a wide range of sugars as a result of their physiological and biochemical characteristics. Molecular identification of *L. plantarum* is however, a new technique in molecular biotechnology. The use of 16Sr DNA genes and their subsequent nucleotide sequencing would help to resolve the ambiguity and inconsistency that is usually experienced in phenotypic characterization technique. According to Olaoye and Onilude, (2009) and Hugas *et al.* (1993), the real identities of the organisms are usually revealed by this method. The molecular method used in this work further confirmed the identities of *L. plantarum* that were used for the fermentation studies. The result obtained is similar to that of Olaoye and Onilude (2009) who reported that the molecular method of identification and characterization of microorganisms have been preferred over the classical ones which make use of the biochemical reactions and proteolytic activities of the organisms. This method is, however, able to solve the problem of ambiguity and inconsistency that is usually encountered in the sugar fermentation techniques. A combination of different approaches in the identification of the organism isolated offer a solution to the ambiguity in the use of the conventional method that make use of the ability of LAB to produce acid from carbohydrate only. The advantage of the molecular method over the conventional one is that it is specific and rapid. However, Jensen *et al.* (1993) opined that DNA finger printing by various PCR methods have been

suggested as clue in tackling the problems that have been associated with phenotypic means of identification.

The highest number of LAB isolates was obtained from local varieties of sorghum (60%), while 40% of the total organisms isolated were from commercially-hawked *Ogi* samples. Only 20% were isolated from the typed varieties Ex Kano, Samsorg 40 and Samsorg 41. The local varieties of *ogi* were produced in the LAB under a strict hygienic and environmental condition. On a comparative basis relative to sorghum, maize and typed, the highest number of organisms were isolated from sorghum (54.2%), followed by maize (29.2%) and the typed varieties (16.7%). The differences in the microbial population are likely due to the differences in the varieties of the cereals or the genetic composition of the cereals.

The typed varieties had the least percentage occurrences of LAB. It was not easy to isolate organisms from the typed varieties like the other samples. Reasons due to the genetic composition of the samples because they have been 'typed' or genetically manipulated. Typed varieties of samples usually have their genetic composition altered as a result of the genetic manipulation done on them. The result obtained is in conformity with that of Sharma *et al.* (1995) on varietal differences of cereals and legumes which are affected by different processing, planting, preservatives, harvesting practises and genetic manipulations. First, it may be for improved seedlings for maximum profit, ability to fight diseases, early maturation, improved storage or shelf life or any other desirable attributes (Onigbinde and Akinyele, 1983).Otherwise, it may be due to the chemicals that were used to preserve the samples (FAO, 1995). In a similar manner, Modu *et al.* (2004) reported that varietal differences of cereal affect the nutritional acceptability of different food made from different millet varieties.

Furthermore, of all the organisms isolated, only nine species of *Lactobacillus plantarum* from various sources were chosen for further work. The selection was based on the following criteria: first, production of alpha and beta galactosidase enzyme and other enzymes in abundance. Secondly, the production of antimicrobial substances: diacetyl, hydrogen peroxide and lactic acid in abundance. Thirdly, the presence of plasmid and production by the isolates, ability to perform better in various physiological processes carried out by the isolates. Their identities were also confirmed by molecular methods of characterization using 16Sr DNA. From these nine, they were further reduced to three denoted *L.plantarum* LV1, *L.plantarum* LV2

and *L.plantarum* LV3. These organisms that were isolated from local sources were found to perform better than all the isolates in all the various tests done. The result obtained is in conformity with that of Sharma and co-worker (1995) on varietal differences of cereals and legumes and Roem *et al*. (2007).

Lactobacillus plantarum have been observed as a dominant LAB in fermented foods used in this work. The observed predominance was found to be in agreement with the work of Onilude *et al.* (1993), Olsen *et al.* (1995) and Wakil and Onilude (2009). The work also showed that *L. plantarum* was the one with the highest frequency. Other LAB were also isolated but *L. plantarum* was preferred because it performed better than other isolates in all the various tests that were carried out. This is in accordance with the work of Johnson *et al.* (1995) that stated that the amylolytic *L. plantarum* accounted for a great percentage of the total LAB isolated from traditional fermented foods. Also, they found out that amylolytic *L. plantarum* strains accounted for 14% of total LAB isolated from *Ogi*. Oguntimehin (1993), Olasupo *et al.* (1993) and Kunene *et al.* (2004) have also described *L. plantarum* as being able to perform better than other LABs in various tests they carried out.

The result obtained is also in conformity with the work of Hammes and Tichazek (1994) who reported the modern concepts or perspectives of the application of LAB especially *L. plantarum* as a good starter with the following characteristics: Selection of the best adapted and safely performing LAB strains. , selection of strains with probiotic effects, selection of strains with health-promoting effects (e.g. production of vitamins or essential amino acids, anti-tumour activity), selection of strains with food protective activities (inhibiting spoilage or food pathogens). These strains can be added to food or used as starters in food fermentations. They may be found as wild-type organisms or can be obtained by genetic engineering according to Hammes and Tichazek (1994).

The various physiological studies shows that *L. plantarum wa*s able to metabolise different sugars as sources of carbon for growth. Utilization of glucose was the highest; followed by lactose and raffinose the least. This is in agreement with the work of Mossel and co-workers (1995) who reported that all LAB ferments glucose with or without the production of gas. Maleata (1998) also stated that *L. plantarum* ferments different sugars at varying degrees with glucose taking a lead. The various LAB were able to utilize different carbon sources for growth – glucose,

lactose and raffinose and enzyme production. This observation agrees with the work of Jones (1999) which stated that LAB are able to utilize different sugars as their main source of carbon to produce energy which they use for growth and metabolite production. This however, could be linked to the ability of these organisms to ferment different sugars, glucose, lactose and raffinose as stated earlier in a complex medium of growth. According to Lealem and Gashe (1994), the different sugars are important component of microbial nutrition which are used for growth, energy and metabolite production. The organisms metabolise glucose and lactose better than raffinose, but the ability of *L. plantarum* to metabolise raffinose is a desirable attribute. This is in agreement with the findings of Reid and Abratt(2005) and Nelson and Cox (2005) on microbial nutrition and metabolism. He reported that LAB metabolise raffinose through the Embden Meyerhof Pathway.

On utilization of sugars by cured and uncured *L. plantarum*, the uncured isolates utilized the sugars for growth more than the cured isolates. However, the organisms utilized glucose better that the other sugars, lactose and raffinose, they were also found to utilize lactose better than raffinose. Glucose also favour the production of all the enzymes especially amylase and alpha-galactosidase than other sugars. This is in agreement with the work of Gomez and Maleata (1998) that stated that LAB ferment the oligosaccharide raffinose in a very weak or slow manner. Storey *et al.* (1988) and Gray *et al.*, (2012) also confirmed this also by describing raffinose as a trisaccharide composing of galactose, fructose and glucose. The inability of some microorganisms in humans to metabolize this sugar is, however, the onset of the problems of gas formation (Thompson, 1998). The result obtained from this work shows that *L. plantarum* was able to break down the complex sugar to reducing sugars and thereby making it available to man in a form that can be absorbed by the body.

The production of the antimicrobial agent, lactic acid, increases as the period of incubation increase from 24 hrs to 72 hrs while diacetyl and hydrogen peroxide reduces with the period of incubation. This shows a direct relationship between the time of incubation and the amount of these metabolites produced. These findings in agreement with the work of Sanni and co-workers (1995) who reported an increase in lactic acid production with increase in the period of incubation and a decrease in diacetyl and hydrogen peroxide as incubation time increases. This however shows that LAB are important in bio- preservation of food and inhibition of pathogenic

microorganisms (Messens and De Vuyst, 2002).The importance of production of Lactic acid, diacetyl and hydrogen peroxide by the selected *L. plantarum* play a major role in their inhibitory action against unwanted organisms. This is in agreement with the findings of Messens and De Vuyst (2002) on antimicrobial properties of LAB. The antimicrobial properties are able inhibit pathogenic and spoilage microorganisms that are associated with food.

LAB lowers the pH of the fermenting medium in which they grow thereby making it unsuitable for the growth of other *Enterobacteria* (Oguntimehin, 1993). This however, is a solution to the problem of poor hygiene and contamination that is usually encountered in weaning foods. Furthermore, the production of the antimicrobial agents like lactic acid and diacetyl increases with fermentation time, the main reason why complete fermentation should be allowed to take place (Sanni *et al.,* 1995; Messens and De Vuyst, 2002). The reduction of pH usually observed in fermentation of cereal is however, a desirable quality in weaning food because the dominance of acid tolerant lactic acid bacteria is able to eliminate the undesirable and pathogenic microorganisms (Oyewole and Odunfa, 1990). Furthermore, for complete fermentation to take place, the cereal must be soaked for at least 5 days. This will allow the LAB to produce the desired flavour, aroma, texture, taste and increased shelf life of the fermented product. This is in accordance with the work of Kunene and co-workers (2004) who opined that an incomplete fermentation of various foods will not produce the desired end result. According to Odunfa (1985), these qualities are usually desired in this fermented cereal. Also, during this period of complete fermentation, the process of souring takes place, this is usually desired in this gruel.

The medium in which LAB is cultivated usually affect the production of metabolites or enzymes by the organisms. In a similar manner, Olaoye and Onilude (2009) reported that the production of antimicrobials by different strains is affected by the composition of the medium for the organism to be chosen as a good starter culture. This is because the components of the medium may differ slightly from one to the other. *L. plantarum* is one of the most widespread strains of LAB used in food technology. According to Pedersen and co-workers (2005), the organism has a homofermentative metabolism and depending on the growth rate, temperature, pH level of substrate and oxygen in the medium, it can also produce acetic acid and acetoin. As a result, the medium of growth need to be supplemented with yeast

extract, a source of vitamin and essential amino acids and nucleic acid. The growth, kinetics, metabolite production, vitamins and amino acid requirement of *L. plantarum* have been studied relative to its use in food fermentation. The proteolytic system of LAB have been described and the physiological knowledge regarding the utilization of carbon and nitrogen sources, amino acid ,mineral requirement and peptides are usually studied for targeted optimization of the organism. The observation recorded by Pedersen *et al*. (2005) is similar to those obtained in this work. The medium of growth was supplemented with various anions and cations and $MgSO_4$ and Triammonium citrate was found to produce the best result in terms of growth of the organisms.

Moreover, physiological studies involving the use of cured and uncured organisms in raffinose metabolism were monitored in relation to raffinose metabolism. The study showed that there was a drastic reduction in the growth and enzyme production of the organisms being monitored. Plasmid curing affected the ability of these organisms to grow in these various environmental conditions and production of enzymes. This observation is in agreement with the work of Oguntimehin (1993) who stated that LAB grows at optimum temperature and conditions of growth. At, adverse environmental conditions, which is thus implicated in different methods used for curing, the organisms did not grow well, as a result, they cannot produce metabolites. This is in accordance with the opinions of Giraud *et al.* (1991) and Messens and De Vuyst (2002). Furthermore, Ilori and co-workers, (1995) reported that at a very high temperature above 60^0C, LAB will not grow nor produce enzymes. This was observed in the physiological studies on the organisms isolated from this work.

This work also showed that plasmid curing reduced the ability of *L. plantarum* to metabolise raffinose and other sugars used in this work. When the cured isolate were used in the various physiological processes, the growth of the organisms were rather very slow. This is in agreement with the work of Gonzalez and Kunka (1985); Lordish *et al.* (1999) and Akano *et al.* (2000) that reported that the ability of LAB to ferment sugars which are commonly found in plants is a selective advantage. The presence of plasmids however governs the fermentation of carbohydrates in LAB. This is of utmost importance in the food fermentation industry. Kondo *et al.* (1985) also reported that the utilization of raffinose by another

LAB, *P. pentosaceus* is linked to the activities of plasmid DNA present in the isolate.

Since LAB are fastidious organisms, the media that they grow in are usually supplemented with anions and cations to meet their complex nutritional requirements. According to Dugas (2004), they require accessory growth factors that can meet their nutritional demand and biosynthetic capabilities. $MgS0_4$ was preferred by the organisms than $FeS0_4$ and $ZnS0_4$ because it meets their demand for minerals salt and growth requirement (Nout *et al.*, 1989 and Pelczar *et al.*, 1993). Also, the anion triammonium citrate was preferred by the organisms to NaCl and $MgCl_2$ from the result obtained in the physiological studies of the isolates. Pelczar *et al.* (1993) stated that the growth of LAB isolates in the presence of different inorganic salt form complexes that is produced by the organisms. The various mineral salts or anions and cations added to the medium of growth improved the performance of the organisms. This made them performed well when their physiology and growth were monitored and they were compared with the ones in which the mineral salts were not added. Optimization of the minimal basal requirement of growth showed that when the medium of growth are supplemented with accessory growth factors, *L.plantarum* performed better. According to Obello *et al*. (1992) and Dugas (2004), an appropriate culturing medium must however contain a carbon source, nitrogen source, anions, cations, and mineral salts. As carbon sources in the growth of *L.plantarum*, glucose, lactose, raffinose and other sugars can be used, also glycerol and sodium acetate can be used. As nitrogen sources in the growth of *L.plantarum*, inorganic and organic nitrogen compounds, such as meat extract, yeast extract, peptone, amino acids, casein hydrolysates, soybean flour and ammonium salts can be used. The pH range for culturing is from is preferably from 5.0 to 5.5, the temperature is comprised between 30^0 C and 40^0C. The enzymes and other metabolites production can be boosted by the addition of these supplements.

Agrawal and Dutta (1987) also reported that the activities of microorganisms are enhanced by the presence of metal ions like Mg^{2+} and Na^+ in the growth medium.

Keith (1992) in his work reported that microorganisms require free metals for their activity such as the ones stated above, it is therefore necessary to ensure that the metals are in sufficient amount in the medium of growth of the isolates. Despite all these, the cured *L. plantarum* showed a reduction in growth, this is because plasmid

curing has affected the ability of the organisms to utilize the metals and the ability to metabolise the growth factors that are required by the organisms. There was however, a sharp reduction in their physiological characteristics and growth as a result of this. As stated earlier by Mark *et al.* (1996) the selective advantage that is conferred on the organisms by plasmid presence is usually lost during plasmid curing.

The optimum temperature and pH required for the growth of LAB isolates as stated by Ilori (1995) and Oguntimehin (1993) is at temperature of 30^0C and a pH of 5.5. The uncured organisms *L. plantarum* grew well at this temperature and pH in the presence of raffinose. A lower temperature or pH did not favour the growth of the organisms. At optimum temperature and pH, uncured *L. plantarum* performed at their best, while there was reduction in growth at very high/low temperature and pH which affected the production of metabolites by the isolates. This was also reported in the work of Messens and De Vuyst (2002) who observed that the growth of LAB are usually affected by extreme environmental conditions. Plasmid curing however, affected the ability of the organism to grow well and utilize the various carbon sources for growth even at the optimum environmental conditions, this is in agreement with the work of Bruce *et al.* (2002) that opined that plasmids are easily lost at extreme environmental conditions and as a result the organisms will not grow well or perform at their best.

The growth of and raffinose utilization by the organisms at different time intervals was also affected by the presence of plasmids; there was an increase in growth from 24 hrs, 48hrs, 96 hrs and 120 hrs using uncured *L. plantarum* . This is confirmed by the work of Wakil and Onilude, (2009) and Agati *et al.* (1998) which states that there is usually an increase in growth in the microbial population as fermentation time increases. Curing brought a significant reduction in growth at different time interval. In a similar manner, Ahrne and Molin (1991) reported that spontaneous changes usually occur in the physiology of the *L. plantarum* during curing.

Peptone favours the growth of *L. plantarum* best of all the nitrogen sources used. Peptone is usually added to the fermenting medium of growth of LAB because LAB are fastidious organisms and they have complex nutritional requirements. Peptone is an accessory growth factor that is usually involved in their medium of growth (Dugas, 2004). This is why they are usually cultivated on media containing

these constituents. The various physiological studies were therefore carried out at different concentration of sugars. This is because the distribution of LAB is related to their high demand for nutrients and their energy type. LAB obtain energy from the metabolism of sugars and fermentable compounds (www.scielo.org.ve/scielo.php). The utilization of nitrogen sources by *L. plantarum* isolates were also considered in the choice of organism for fermentation. The ability of *L.plantarum* to grow at varying levels of different temperature, pH, carbon sources, nitrogen sources, anions and cations concentration is an important factor that is usually considered in the choice of a good starter culture for food processing. This observation is similar to those reported by earlier workers such as Olaoye *et al.* (2009) and Onilude *et al.* (2009).

The result obtained from this work showed that *L. plantarum* produced α-galactosidase more than the other isolates (Table 4.7).This agrees with the work of Mitul *et al. (*1973) who also described *L. plantarum* as having the ability to produce the enzyme more than the other *Lactobacilli*. This however, was chosen as criteria for selection of the organisms to be used for further work. It was observed that its production was more in the local varieties (1.8 unit/ml) than commercial (1.2 unit/ml) and typed varieties (1.1 unit/ml). This is in agreement with the work of Storey *et al.* (1998) and Thompson *et al.* (1998) that implicated *L. plantarum* as producing α-galactosidase in abundance under optimum condition of growth and metabolites production of the isolates (pH 5.5 and a temperature of 30^0C). Alpha-galactosidase is an enzyme that is possessed by few groups of microorganisms and LAB that metabolize the oligosaccharide raffinose. This enzyme is not present in humans but it is produced by some micro organism which is able to break the long polymeric chain present in the complex sugar raffinose (Alcamo *et al.*, 2000). As a result of this, the enzyme makes it available in a form that is metabolisable by man.

Production of the enzyme α-galactosidase is a desirable quality of *L. plantarum* that was harnessed in this work. According to White and *et al.* (1985), α-galactosidase enzyme is known to break down raffinose at the α-1, 4 glycosidic bond while β-galactosidase is able to breakdown the chain at the β-1, 6 positions. The production of α and β galactosidase by *L. plantarum* was employed to break down the raffinose oligosaccharide that is present in soybeans through fermentation. Also, Rodriguez *et al.* (2009) opined that *L. plantarum* is the commercial starter most

frequently used in the fermentation of food products of plant origin because of its ability to produce the enzyme α-galactosidase .

The work of Hammes and Tichazek (1994) reported the modern concepts or perspectives of the application of LAB to include the following: Selection of the best adapted and safely-performing LAB strains in physiological processes, selection of strains with probiotic effects, selection of strains with health-promoting effects (e.g. production of vitamins or essential amino acids, anti-tumour activity), selection of strains with food protective activities (inhibiting spoilage or food pathogens). These strains can be added as food or used as starters in food fermentations. They may be found as wild-type organisms or can be obtained by genetic engineering (Hammes and Tichazek, 1994).

Production of various enzymes by the *L.plantarum* isolates were also more in samples that were isolated from the local varieties. These organisms had the highest growth rate and enzyme production rate. The organisms from the local varieties produced the various enzymes in abundance than the organisms that were isolated from the commercial and typed varieties. Amylase, for example had the highest production in the local verities, this was followed by enzyme production in the commercial sources and that of typed varieties was the least. Mellibiase, invertase, beta-galactosidase enzymes also followed the same trend. The observation conforms with the work of Wallestin (1987), Reid and Abratt (2005) and Nelson and Cox (2005) who opined that the best result for enzyme production were obtained at optimum growth rate. Since, the organisms from the typed varieties did not grow well, enzyme production capacity also reduced greatly in them. The production of α and β- galactosidase, invertase, mellibiase and amylase enzymes were thus abundant in samples that grow well. The production of crude protein and reducing sugar was also affected by the principle of increased growth favouring increased enzyme production. According to Reid and Abratt (2005) and Nelson and Cox (2005), the optimum condition of growth of microorganisms and metabolism also favour optimum metabolite production.

The production of enzymes and other metabolites like crude protein and reducing sugars were also affected by plasmid curing of *L. plantarum*. There was a drastic reduction in the amount of enzymes produced using cured isolates when compared with the uncured isolates. α and β- galactosidase production reduced considerably in the cured when compared to the uncured. This trend was also

observed in the amylase, mellibiase and invertase production of the isolates. Also, crude protein and reducing sugar production of the isolates reduced in the cured when compared to the uncured in all the samples as a result of plasmid curing. The results obtained are in conformity with the work of Lordish *et al.* (1999); Akano *et al.* (2000) and Bruce *et al. (*2002) who stated the importance of plasmid presence in organisms for optimum production of metabolites. The presence of plasmids in an organism confers a selective advantage on such organism. For instance, the presence of plasmids have been implicated in the production of proteins (Bruce *et al.*, 2002; Prescott *et al.*,2008) whose bonds are easily broken at extreme environmental conditions. The presence of plasmids in *L. plantarum* thus confers on them valuable characteristics that could be used in the field of biotechnology. Curing affected the ability of *L. plantarum* to produce these enzymes as there was reduction in the production of these enzymes. The production of α and β- galactosidase, amylase, mellibiase, and invertase enzymes were affected by plasmid curing. Also, the production of crude proteins and reducing sugar was affected negatively by plasmid curing. The result is similar to those obtained by Ahrne and Molin (1991) who reported that spontaneous changes usually occur in the physiology of the organism during curing.

Enzymes are proteins that are destroyed at high temperature; the process of curing is however, carried out at extreme environmental condition which does not favour the production of such enzymes. As a result there was a reduction in the quantities of various metabolites produced by *L. plantarum* isolates. This is also in agreement with the work of Mark *et al.*(1996) who stated that plasmids are sometimes lost at extreme environmental conditions by organisms in which they are found. As a result, the selective advantages they confer on the host organisms are usually lost.

The total oligosaccharide content and the different types of raffinose family of oligosaccharides (RFO) present in raw, cooked and roasted soybeans were analysed. The RFO observed were raffinose, stachyose and verbascose. The analysis also revealed the reducing sugars sucrose, glucose and raffinose that were produced during the breakdown and hydrolysis of the RFO by fermentation. Stachyose was found to be the predominant sugar in soybeans. This was followed by Raffinose and Verbascose. Similar results were reported by Revilleza *et al.* (1990); Sani *et al. (*1997) and Oboh *et al*. (2000).

The effect of processing on the sugars and total RFO content of the legume revealed that cooking in water and roasting resulted in a loss of RFO to an extent when compared with the raw samples. The results agree with those earlier reported by Burbano *et al*. (1990) and Hymowitz (2012) who established that oligosaccharide content of legumes was influenced and reduced by different pre-treatments methods and environmental factors. But fermentation is able to reduce it to a level that is safe for consumption. Both processes involved the use of heat. Hence, the reductions may have been due to heat induced hydrolysis of the oligosaccharide to simple disaccharides. These findings are in agreement with those reported for cowpeas and other legumes in the work of Onigbinde and Akinyele (1983); Somlari and Balogh (1993) and Oboh *et al*. (2000). Cooking also resulted in slight loss of sugar which is in agreement with the result of Rao and Belavady (1978) and Uzogeru *et al.* (1996). Similar report was also observed by Omafuvbe *et al*. (2007) who reported that the low concentration of reducing sugar in the dehulled beans at the onset of fermentation is an indication that some sugars may have been leached out in the soak water.

Fermentation for 120 hours with *L. plantarum* reduced the total RFO content of soybeans to different levels in the samples. Losses of 30%, 28% and 37 % in stachyose, raffinose and verbascose respectively were observed in the fermented raw samples. A 72%, 58% and 41% reduction in stachyose, raffinose and verbascose respectively was observed in the fermented cooked samples. Fermented roasted samples showed a 76%, 68% and 71% reduction in stachyose; raffinose and verbascose respectively were observed when compared to raw seeds. These losses were significant at the different fermentation time. This is also similar to the findings of Mulimani and Ramaligam (1995) who reported complete hydrolysis of stachyose and raffinose to more than 60% hydrolysis due to fermentation.

The reduction of RFO in the raw sample was small when compared to the other samples. This may be as a result of the relative hardness of the seed coat which limits the uptake of water and may prevent thorough fermentation of the samples. Significant reduction in the RFO content has been reported during fermentation and other forms of pre-treatment by different authors (Akinyele and Akinlosotu, 1991; Abdel-Gawad, 1993). The pre-treatment was however necessary to make the samples amenable to LAB fermentation .This is because it was not easy to ferment the raw samples with the hard seed coat but the pre-treatment methods have solved

this problem. Similar finding was also reported by Shalini (2006) and Hymowitz (2012).

The changes in total RFO content due to fermentation with *L. plantarum* indicated a significant reduction in all the samples due to the metabolic activity of *L. plantarum. S*imilar findings have been reported for related leguminous plants such as cowpea, African yam beans, Jack beans and Pigeon peas by the following authors Onigbinde and Akinyele, (1983); Revillez (1990); Akinyele and Akinlosotu, (1991); Abdel-Gawad (1993), and Oboh (2000) . The relative reduction in the RFO observed in the study could be attributed to the presence of both alpha and beta-degradation of the sugars at alpha 1, 4 and beta - 1, 6 positions, respectively. This is similar to that obtained by Oboh and co-workers (2000). This is because *L. plantarum* produce the enzyme alpha-galactosidase in the medium of growth. This is able to breakdown the oligosaccharides of the RFO. The enzyme alpha-galactosidase is able to cleave galactose from raffinose, stachyose and Verbascose selectively leaving behind sucrose and other reducing sugars. This is in accordance with the observations of workers like Kuo *et al. (*1988); Price *et al. (*1988); Rataria *et al*. (1990) and Muzquiz *et al.* (1993).

A reduction rate of about 20% was observed in all the sugars at every 24 hrs up to 96 hrs. This is similar to the findings of Leblanc *et al*.(2004) who reported that *L. plantarum* was able to eliminate raffinose, a nondigestible alpha-oligosaccharide (NDO) found in soy products showing a consumption rate of 0.25 g l(-1) h(-1) (pH 6.0-5.0). The removal of raffinose was due to the high alpha-galactosidase activities of these lactic acid bacteria, which was highest at pH 5.5 (5.0 U/ml). The combination of the various pre-treatment methods and fermentation of soybeans have caused a reduction in the RFO and ANF. This has led to increase in the nutritional composition of the food in which it is added, it also increased the availability of minerals, for example Ca^{2+} and Fe^{2+} .This observation are in agreement with earlier studies by Onilude *et al*. (1999); Sanni *et al*. (1999) and Wakil and Onilude (2009).

There was, however, a relative increase in the reducing sugars profile, a 61%, 53% and 32 % increase was observed in the glucose, fructose and sucrose after the raw samples were fermented with *L. plantarum.* The fermented cooked samples experienced a 78%, 64% and 44 % increase in glucose, fructose and sucrose respectively while the fermented roasted samples experienced a % increase of 87, 71

and 52 respectively during fermentation of all the samples with *L. plantarum* from 0- 96 hrs. Similar findings was reported by Rataria *et al*.(1990) and Muzquiz *et al.*(1993) who reported increase in the reducing sugar profile as the total RFO reduced due to its breakdown during fermentation by the enzymatic activities of microorganisms. The reducing sugar in the fermenting soybeans increased from 24 hrs to 96 hrs and decreased thereafter in all the fermentation procedures. The period of rapid increase coincides with the period of increased total reducing content, reduced RFO, and alpha and beta galactosidase activity. There was a reduction at 120hrs. The decrease in reducing sugar level of the fermenting powdered samples indicates that they are being used by *L. plantarum* for metabolism. Similar result was reported by Omafuvbe *et al*. (2007).

Storey *et al.* (1998) and Gray *et al.,* (2012) reported that complete hydrolysis of raffinose gives 1 mole each of galactose, D-glucose, and D-fructose. Galactose is in turn broken down to glucose and sucrose. This explains why the percentage of the reducing sugar produced is highest in Glucose in all the samples: raw, cooked and roasted. He also reported that raffinose can be hydrolyzed by enzymes in two ways. Invertase (β-D-fructofuranoside) hydrolyzes the raffinose part of the molecule to give galactose and D-fructose. According to Reid and Abratt (2005) and Nelson and Cox (2005), it contains the enzyme α-D-galactosidase, which hydrolyzes the galactose residue to yield D-glucose and sucrose through the Embden- Meyerhof pathway. Moreover, metabolic pathways of biosynthesis or degradation of RFO compounds in soybeans by *L. plantarum* have been described.

As observed in the work, the total oligosaccharide decreased as the fermentation time progressed from 0-120hrs producing simple sugars at the end of the metabolic pathways. *L. plantarum* was able to produce this enzyme in abundance and as such breakdown the raffinose. The works of Barker (1997) and Gray *et al.*, (2012) corroborate these findings. They demonstrated that the metabolism and utilization of raffinose by lactic acid bacteria goes through a 2-stage process. First, raffinose is broken down into sucrose and galactose in the first stage reaction. While in the 2nd stage, the galactose produced is further broken down into fructose and glucose by the metabolic activities of LAB.

The use of intracellular alpha-galactosidase from *L. plantarum* to breakdown raffinose, stachyose and verbascose in raw, cooked and roasted soybeans was achieved. The optimum conditions for the enzymic hydrolysis of raffinose, stachyose

and verbascose was pH 5.5 at 50^0 C for 24hrs to 96 hrs. Alpha-galactosidase showed optimum activity at pH 5.0 and 50^0 C, with the substrate p-nitrophenyl-alpha-D-galacto-pyranoside (PNGP). The enzyme activity showed detectable loss in reducing sugar production after 96hrs. This is also similar to the findings observed in work of Mulimani and Ramalingam (1995) on enzymic hydrolysis of raffinose and stachyose in soymilk by alpha-galactosidase. They reported the optimum activity of the enzyme at pH 5.0 and 50^0_C and no loss of activity was observed at this environmental condition

The result of this work shows that cooking reduces the Antinutritional Factors (ANF): Tannin, phytate, protease and trypsin inhibitor by (10%). This is in agreement with the work of Jansen *et al.* (1980) *and* Jood (1985) who reported a slight loss in the ANF of soybean during cooking and soaking in water. Roasting, however, reduced the ANFs by (40%). Similar observations has been noted in the work of Lopez and Gordon (1989), Salunkhe and Cheran (1990) and Oboh *et al.* (2000) all of who opined that roasting at a temperature of 100^0C in a hot air oven in the laboratory reduced the ANF in different legumes to about 50%, a level that may need to be further broken down for it to be safe for human consumption when compared with the raw soybeans samples. Oboh *et al.* (2000) reported that local methods of processing substantially reduced the raffinose family of oligosaccharide in grain legumes particularly to a level that is safe to be consumed by infants, while the ROF are degraded by alpha-galactosidase; sucrose is left behind in the fermenting medium thereby increasing the nutritional composition.

Fermentation is also one of the ways of reducing ANF. There was observed a significant reduction in the tannin, phytate, and protease and trypsin inhibitor content of soybeans when the various pre-treatment methods mentioned above were used while lactic acid fermentation reduced it to about 60%. As observed in this work, fermentation reduced the ANF to almost nil, a level that is safe for it to be used as supplement in weaning foods. This is in agreement with the work of Khetarpaul *et al.* (1991), Ado *et al. (*1995), Onofiok and Nnayelugo (1998) and Hymowitz (2012) who reported decreases in the antinutritional composition of different legumes used as complementary weaning food during fermentation. They also reported an increase in the nutritional composition of the cereal-based legume weaning food during fermentation.

Results obtained in this work revealed that, *L. plantarum* was able to reduce the ANFs in soybeans to a very safe level that make it useful as a good weaning food. Similar observations have been reported by Kheterpaul (1989), Livingstone *et al. (*1993) and Wakil and Onilude (2009). The reduction of ANFs in soybeans by *L. plantarum* during fermentation is a desirable quality of the organisms that can be harnessed in the formulation of good weaning diet for infants. Curing of *L. plantarum* also affected its ability to reduce the ANFs . The cured isolate was only able to reduce the ANFs during fermentation slightly to a level not safe enough for consumption. This is because the result obtained only showed a decrease that is not significant enough to notice any detectable reduction in the raw samples when compare to the fermented ones with cured *L. plantarum* (Tannin reduced from 2.0 to 1.5mg/g, phytate from 1.2 to 1.0mg/g, trypsin inhibitor from 1.2 to 1.1mg/g and protease inhibitor from 1.2 to 1.1mg/g). This was corroborated by the work of Salunkhe *et al.* (1990) and Amankwah *et al.* (2012). Ability of *L. plantarum* to reduce the ANFs is a plasmid- linked trait because its ability to reduce the ANFs got lost during curing. Lordish *et al.* (1999), Akano et *al. (*2000) and Bruce *et al.* (2002) reported that microorganisms lose the special and desirable ability conferred on them by the presence of plasmid during curing.

Furthermore, fermentation of cereal through microbial activity led to the hydrolysis of the RFO and reduction of ANF. These reduced with fermentation time which could be due to the breakdown and degradation of oligosaccharide to smaller units by the action of the enzymes mobilized during the fermentation period and the activity of alpha and beta galactosidases while the reducing sugars and nutritional composition of the blend increased with fermentation time. These observations agree with the earlier reports of Khetarpaul *et al.* (1991), Ado *et al. (*1995), Onofiok and Nnayelugo, (1998) and Amankwah *et al.* (2012) on cereal and legumes based gruels.

Provision of nutritionally adequate, cheap, easy- to- prepare, one with good health benefit for the infant and prolonged shelf life is a major challenge in West Africa. Weaning food is usually required when breast milk alone cannot meet the nutritional requirement of a young child. There are commercial weaning food in the market but they are usually very expensive and not within the reach of a common man. Some of these weaning foods however have their various challenges. Some that are prepared at domestic level contain Raffinose oligosaccharide that causes abdominal pain, bloating and flatulence in young children, while some also contain

some anti nutritional factors as a result of poor method of preparation, others may not be prepared hygienically. These problems were addressed in this research with the metabolic activities of *L. plantarum* through fermentation. This is in accordance with the work of Mariam (2004) and Macdonald *et al*. (2012) on preparation of a good weaning food and its associated problems.

Food fortification or supplementation is the public health policy of adding micronutrients (essential trace elements and vitamins) to foodstuff to ensure that minimum dietary requirements are met (Mariam, 2002) Nagai *et al*.(2012).and Addition of micronutrients to staples and condiments can prevent large-scale deficiency disease in these cases and prevent the attended cases of malnutrition that is associated with using cereal based diet as weaning food (Malleshi *et al.*, 1989; Macdonald *et al*. (2012). One of such is the use of soybeans as fortification or supplementation in weaning foods. Several authors have suggested the use of soybeans in the formulation of a good weaning diet and the observations in this work are in conformity with such earlier reports. Authors like King *et al.* (1993), Ado *et al.(*1995), Badamosi *et al.(1995),*Onofiok and Nnanyelugo(1998), Mariam (2004), Onilude *et al.(*2004) and Wakil and Onilude (2009) have made such reports. The presence of raffinose oligosaccharide and some antinutritional factors in soybeans has limited its use as a good source of protein in Nigeria despite its several advantages, and according to Castillo *et al.* (1990) and Nagai *et al*. (2012). , the cereal can be consumed by infants when the RFO and ANF have been removed successfully.

Furthermore, the nutritional composition of soybeans was enhanced by fermentation using uncured L. plantarum. This is in agreement with the work of Svanberg (1992) and Onilude et al. (2004) and Nagai et al. (2012). The ANF was also reduced by fermentation, a result which agrees with the work of Afoakwa, (1996) and Wakil and Onilude, (2009) and in the various soybeans samples, even thou roasted soybeans samples was preferred in the formulation of the food weaning blend. This result because cooking for example, reduced some of the nutritional composition. This in agreement with the work of Onigbinde and Akinyele (1983) and Amankwah et al. (2012) that some nutrients are leached into water during cooking. Also, soybeans is not usually consumed raw except with some form of pre-treatment methods such as soaking, dehulling, milling, cooking and roasting (Amankwah *et al.* (2012);Nagai et al. (2012).

Fortification of sorghum *ogi* improved the nutritional composition of the cereals. This is in agreement with the work of Akinrele (1971) and Campbell-Platt (1994) that stated that cereals are deficient in lysine, but are rich in cystein and methionine. Legumes on the other hand are rich in lysine but deficient in sulphur containing amino acids. Thus by blending cereals with legumes, the overall protein quality is improved in the blend (Amankwah et al. (2012); Nagai et al. (2012; MacDonald *et al*., 2012).

.On nutritional composition, the increase in the quality of the food blend by the addition of roasted soybeans flour was significant ($p<0.05$). The result is consistent with other report on the improvement in quality of corn protein by protein complementation or supplementation as determined by more traditional evaluation methods and as observed by workers like Akinrele and Edwards (1971); Adeniji and Potter (1976), Ekpenyon *et al. (*1997), Onilude *et al*. (2004) and Wakil and Onilude (2009).There was an increase in protein, ash content, ascorbic acid, thiamine, riboflavin, niacin and mineral content. This is similar to the work of Plahar *et al.* (1993) who reported that when *ogi* is fortified with a high quality vegetable protein such as soybean flour, the fermented meal could serve as a major source of protein, especially in weaning foods for infant. Ado *et al.* (1995); Amankwah *et al.* (2012); Nagai *et al.* (2012; MacDonald *et al*., (2012) also opined that fortifying corn meal with defatted soybean caused a further improvement in the vitamin content of the food blend. Furthermore, King *et al.* (1993); Ighogboja (1992); Gopaldes *et al.* (1988) confirmed this.

The formulation made in this research could easily serve as a good source of weaning food for infants. The traditional fermentation of *ogi* which consist of metabolic activities of *L. plantarum* and inclusion of roasted soybeans which caused an increase in the protein content is however, a desirable quality. The development of weaning food fermentation in developing countries is a great challenge that can be addressed by the use of such weaning food composition. In a similar observation, Akinrele (1970); Akinrele and Edwards (1971);Nout *et al.* (1989) and Macdonald *et al*. (2012) opined that the traditional fermentation which contribute an important role in food supply especially fermented cereal from indigenous raw materials play an important role on daily nutrition in developing countries.

According to Amankwah *et al.* (2012), the widespread problem of infant malnutrition in the developing world has stirred efforts in research, development and

extension by both local and international organizations. As a result, the formulation and development of nutritious weaning foods from local and readily available raw materials have received a lot of attention in many developing countries**.** Malnutrition is a major health problem in developing countries and contributes to infant mortality, poor physical and intellectual development of infants, as well as lowered resistance to disease and consequently stifles development. Protein-energy malnutrition generally occurs during the crucial transitional phase when children are weaned from liquid to semi-solid or fully adult foods. During this period, children need nutritionally balanced, calorie-dense supplementary foods in addition to mother's milk because of the increasing nutritional demands of the growing body (Amankwah *et al.*, 2012).

The formulated blend also meets all the requirements that are usually sought for in developing an adequate weaning food for infants. This agrees with the nutritional composition and specification for home-prepared and commercially-processed food blends as laid down by Protein advisory group of the United Nations Guide (1981) as recommended by World Health Organization and National Institute of Nutrition for children and rural mothers (FAO/WHO, (1995) and (FAO, 1997). It is also able to supply the nutritional and energy requirement of a growing child in the Food and Drug Administration recommended requirement of (2001). It is able to meet the daily nutrient composition of the recommended standard for weaning when compared with commercially available ones. These observations are in agreement with earlier studies by Akinrele and Edwards (1971); FAO (1991), Lathomi and Nnakwe (1995), Modu *et al.* (2004), Mariam (2004) and Macdonald *et al*. (2012).

This is also corroborated by the work of and confirmed by Ekpenyon *et al.* (1997) and Wakil and Onilude, (2009) who reported a significant increase in nutritional composition of fermented cereals when the fermentation time increases. Furthermore, the activities of LAB in foods are confirmed because LAB increases the acidity of the fermenting cereal during the process by lowering of the pH and preventing the growth of spoilage microorganisms (Messens and De Vuyst, 2002; Oyewole and Odunfa, 1990).

Roasting of the soybeans would have reduced the oligosaccharide content to a certain extent. Raffinose intolerance will now be dealt with by the LAB in the guts especially *L. plantarum* when the fermented *ogi* sample is consumed (Prescott *et al.*, 2008).This is how LAB operates. The process of breaking down the oligosaccharide

is done in the guts (Prescott *et al.*, 2008). This has been demonstrated in the laboratory *in vitro* and it can then be translated to everyday use. Owing to the acidic pH of the stomach (2-3) and the gastric content, most microorganisms are killed. The ones that survived include *Streptococcus spp, Lactobacillus spp, Candida spp and Enterococcus spp.* They survived because they are able to tolerate the gastric pH in the stomach. A few also survive in the small intestine because of the influence of the stomach acidic juices and the inhibiting influence of bile and pancreatic secretions (Prescott *et al.*, 2008). Some *Lactobacillus spp* have been found to be associated with the breakdown of oligosaccharide which starts from the mouth and ends in the stomach to cause the metabolism and breakdown of complex food substances.

Another important quality of a desired food weaning blend is one with a low raffinose-oligosaccharide content in which the complex sugar has been reduced to a level that is digestible or metabolisable by the infants as well as making the reducing sugar readily available for energy production. This was achieved in the food blend formulated in this work. With the use of uncured *L. plantarum*, there was a reduction in the oligosaccharide content of roasted soybeans from 0 hrs to 96 hrs. Furthermore, there was an increase in the reducing sugar content of the soybeans during fermentation. This corroborate the work of Espinosa and Puperez (2006), Kim *et al.* (2003) both of which reported a reduction of galacto- oligosaccharide content of soybeans during fermentation. Also, Ado *et al.* (1995) stated a reduction in the oligosaccharides content of soybeans by the presence of enzymes. The reduction of RFO, ANF and other unwanted /toxic substances during fermentation and improvement of the nutritional composition of the food by *L. plantarum* is one of the attributes of a good starter culture. The results of this study agrees with that of LeBlanc *et al.*(2004) on selection of the optimum growth conditions of *L. plantarum* with elevated levels of alpha-gal to be used in the reduction of non- reducing-oligosaccharide in soy products when used as starter cultures (Amankwah *et al.*,(2012; Nagai *et al.*,2012; MacDonald *et al.*, 2012).

As confirmed earlier by this research work, uncured *L. plantarum* produced the enzyme α and β galactosidase that is able to breakdown the raffinose present in soybeans at the α- 1, and β- 1, 6 position as reported by Steinkraus (1985) and White *et al.(* 1985). Giraud *et al.* (1993) also reported that unwanted substances are removed from different food products during fermentation by LAB; for example, the cyanide content of cassava is reduced during fermentation. Also, Lordish *et al.*

(1999) and Akano *et al.* (2000) opined that some organisms including LAB possess metabolic/catabolic plasmids that are able to break down complex sugars into simple sugars. Such organisms are also able to break down difficult components. This is one of the characteristics possessed by *L. plantarum* in breaking down raffinose and such abilities are plasmid-linked.

Curing affected the ability of the *L. plantarum* to improve the nutritional composition of soybeans during fermentation. This is in agreement with the work of Gonzalez and Kunka (1985) that reported the ability of LAB to ferment the trisaccharide raffinose as being linked with the presence of plasmid DNA. Plasmid curing also affected the organoleptic attributes of the weaning food blend. The flavour, texture, appearance, palatability, colour and acceptability are affected by plasmid curing. This is in agreement with Prescott *et al.* (2008) that stated that the genetic information that confers a selective advantage to the organisms is lost during curing; such attributes are desirable in food industry and also in biotechnology. During curing, plasmids are thus destroyed and so are not replicated in the organisms that have them. Furthermore, the work of Mark *et al.* (1996) states that plasmids are replicated during cell division i.e. growth. They are metabolic plasmids or degradative or catabolic plasmids. According to Lordish *et al.* (1999) and Alcamo *et al*. (2000), they carry the genes required to break down complex sugars into simple sugars. They also allow a host bacterium to metabolize normally undegradable or difficult compounds such as various pesticides, chemicals and complex sugars.

Curing affected the ability of these organisms to grow normally and their functions either to confer production of antimicrobial agents, transfer of traits, fertility, production of bacterial proteins, pathogenicity and ability to break down complex sugars (Lordish *et al.*, 1999 and Alcamo *et al.*, 2000) as confirmed by these workers are lost during plasmid curing. Plasmids are also useful tools in research and biotechnology. Utilisation of raffinose by *L. plantarum* isolated from indigenously fermented cereal is a plasmid –linked trait.

5.1 CONCLUSION

Raffinose oligosaccharide's presence in weaning foods is a problem that can be overcome when active, viable and healthy LAB are used for the fermentation of such foods. This was accomplished by the hydrolysis of raffinose by LAB especially *L. plantarum* that was used for the fermentation in this work. The nine isolates chosen for this work however contained plasmid because the plasmid profile was done and it agrees with the work of Alcamo and co-workers (2000).

The presence of trisaccharide- oligosaccharide raffinose, a member of RFO in soybeans also received a good treatment. *L. plantarum* produces enzyme α- and β-galactosidase that are able to break down the foods in which the consumption of raffinose is high. (Gray *et al*., 2012; Hymowitz, 2012). Apart from the discomfort that is usually experienced, it results in vomiting, diarrhoea, weight loss and malnutrition. Because the little child cannot metabolise the complex sugar if consumed on a regular basis, malnutrition sets in. *L. plantarum* was thus able to metabolise the complex sugar and break it down to simple sugars in a manner that is readily available and metabolisable by the growing infants so that they can use such for growth.

Most importantly, the problem of gas production, bloating, abdominal pain and flatulence caused by the consumption of complex sugars and ANFs that is usually encountered by using legumes and other cereals that contains some antinutritional factors was addressed with the use of *L. plantarum* as a major organism implicated in the fermentation of cereal. The reduction of raffinose-oligosaccharides and antinutritional factors was also achieved, such being a major component of legumes and some cereals. The work shows that LAB is able to reduce these and improve the nutritional composition of the weaning food blend.

As demonstrated in this study, the ability of *L. plantarum* to break down raffinose oligosaccharide is a plasmid-linked trait. *L. plantarum* lose their plasmids easily as a result of handling e.g. the use of heat in soaking cereals for fermentation, chemical, poor storage condition of gruels and other environmental factors make them to lose their plasmids easily. Efforts should therefore be directed towards preserving the various qualities that are found in this organism.

L. plantarum obtain energy by their ability to utilize and metabolise the raffinose oligosaccharide. Such nutrients are identified which encode the enzymes

that are necessary for transport of these substrates for their subsequent conversion in glycolysis (genome.Igi-psf.org/draft-microbes/pedpe/pedpe.home.html). *L. plantarum* is able to metabolise the large amount of complex sugars that are present in soybeans and therefore reduce or remove the limited use and the barrier to wide use of the leguminous food plant.

This work has shown the need for the choice of adequate cereal used in fermentation which is able to reduce the problem of pathogenic microbial population in the weaning food. It also solve the problem of malnutrition and poor nutritive value of weaning food by suggesting ways of preparing adequate weaning food blend that will meet the nutritional and energy composition of a growing infant. Cereals and legumes are abundant in Africa and West Africa Sub-region; thus these potentials can be harnessed to prepare adequate weaning foods for children. This can be made into gruels that are fermented with LAB and fortified with soybeans (Amankwah *et al.*, 2012; Nagai *et al.*, 2012; MacDonald *et al*., 2012).

Fermentation should be as natural as much as possible; it should also be carried out under hygienic condition and preparation methods. This will reduce infant mortality rate. Also, preparation of adequate and well balanced weaning food is cheap, easy to prepare and has so many advantages. This work will however, give a reassurance to our nursing mothers who want to give the best to their children. Also, the Millennium Development Goal (MDG) of the UN is being achieved as this will reduce the problem of poverty, malnutrition and infant mortality rate. Also, the nutrient composition of the weaning food blend formulation and protein content agrees with the Codex Committee on Foods for Special Dietary uses (1985) and Protein Advisory Group of the United Nations (1971); as well as comparison with the Manual for feeding infants (Cameroon and Hofvander, 1983). As a result, the food blend can compete adequately with the commercially available infant weaning foods. This was enhanced by the fermentative activity of the probiotic *L. plantarum.*

Therefore, the consumption of fermented foods should be encouraged. Also, preparation of adequate and well balanced weaning food is cheap, easy to prepare and has so many advantages (Amankwah *et al.*, (2012); Nagai *et al.*, (2012; MacDonald *et al*., 2012). According to Messens and De Vuyst (2002) and Onilude *et al.* (1995), we can all harness the ability of *L. plantarum* as a natural food preservative to increase food safety and stability. There should therefore be more awareness and education especially in the rural areas on the roles of LAB in

preparing adequate infant weaning food. With simple fermentation technology, lactic acid bacteria in which *L. plantarum* was implicated have been able to solve the problem of ROF and ANF that is usually associated with food.

The various characteristics of *L. plantarum* described in this work can be harnessed so that the genes of the organisms can be manipulated for suitable purpose in its use as starter organisms and improvement in the food industries and other biotechnological research. Efforts should therefore be directed to this end.

Finally, utilization, fermentation and metabolism of raffinose, a complex oligosaccharide by *L. plantarum* from indigenously fermented cereal gruel is thus a plasmid-linked and plasmid-driven trait and ability. This is confirmed in all the tests carried out in this research work. Plasmids are also useful tools in research and biotechnology. The utilization of raffinose is linked to the activities of plasmid DNA present in the isolate. Raffinose utilization by *L. plantarum* from indigenous fermented food sources could be exploited to bring about reduction in antinutritional factors and oligosaccharide content of soybeans; such utilization was found to be plasmid linked.

5.2 RECOMMENDATION

A desirable characteristic of *L. plantarum* that is required in food biotechnology is the reduction of the RFO that is responsible for the flatulence, abdominal pain and discomfort that is usually experienced as a result of the consumption of these complex sugars. This attribute of *L. plantarum* is however beneficial to the consumers because RFO can be broken down into simple sugars which can be metabolised by the body. It is a nutritionally beneficial characteristic of *L. plantarum.*

The various characteristics of *L. plantarum* described in this work can be harnessed so that the genes of the organisms can be manipulated for suitable purpose in its use as starter organisms and improvement in the food industries and other biotechnological research. Efforts are hereby directed to this end. The use of lactic acid bacteria (LAB) expressing alpha-Gal enzyme especially *L. plantarum* is a promising solution for the degradation of RFO and ANF in soybeans. This could facilitate the possible application of selected bacteria or their enzymes in the elaboration of food products with improved characteristics.

It was, however, discovered from this research that nursing mothers due to their activities and ignorance, denature the organisms, cure the plasmids in them and make them ineffective for the purpose of breaking down this complex sugar. Some of the activities that result in curing of plasmids unknowingly are the use of heat and lack of patience in allowing the fermentation to reach the peak (5 days) before it is stopped. Organisms that are however isolated from such methods of incomplete fermentation do not completely break down the oligosaccharide that are found in such food blend. This however results in gas formation, bloating and flatulence and other associated problems. Also, the commercial hawkers usually use hot water to soak the cereal and this destroys the naturally occurring LAB. LAB are usually destroyed at a very high temperature. The hawkers will not get the best form of gruel if they did not allow the spontaneous fermentation to take place.

Sorghum *ogi* was also preferred for the composition of the weaning food blend because the nutritional composition was more than the ogi made from white and yellow maize. Most of the weaning food formulated in time past by different authors also make use of sorghum *ogi* (Malleshi *et al.*, 1989; Onilude *et al.*, 2004; Wakil and Onilude, 2009; Amankwah *et al.*, 2012; MacDonald *et al.*, 2012; Nagai *et*

al., 2012). In a trial experiment that was done, ratio (i.e. *ogi* 3 and soybean 1) 3:1. 3:2 and 3:3 were tried for sorghum to soybeans. It was observed that fermentation was rather slow and production of reducing sugars was small at higher ratio (1:4 and 1:5). The end product was however, not acceptable because of the strong beans flavour that was impacted by the soybeans. This is why ratio 1:3 was chosen for the fermentation of the food blend. This agrees with the work of Ado *et al. (*1995) who reported that at higher level of soy flour, a strong undesirable flavour was obtained in the mixture. This is also in agreement with the report of Mitzner *et al.* (1984) and Hymowitz *et al*. (2012) on recommended ratio of root crop to legume seeds.

On consumer acceptability, a good packaging is required and also to give the product a good shelf life. This is because *ogi* is cheap and easy to prepare, while roasting of soybeans is also cheap and easy to do. *Ogi* can be preserved on a household level in a refrigerator but where this is not available, it can be left at room temperature and the fermenting water changed regularly. Fermentation should be complete, at least 5 days before milling and sieving. This is consistent with the work of Ado *et al.* (1995) and Amakwah *et al*. (2012) who reported that when fermentation of *ogi* was extended beyond 3 days, protein quality was further enhanced.

Soybeans are considered by many agencies to be a source of protein. A complete protein is one that contains significant amounts of all the essential amino acids that must be provided to the human because of the body's inability to synthesize them. For this reason, soybean is a good source of protein, amongst many others. The combination of this legume with fermented cereal can however solve the problem that is associated with it consumption and limited use. Although it is commonly known that soybeans have health benefits, including one of the highest content of protein for a legume (Jeyaram *et al.*, 2008; Gray *et al*., 2012; Gray *et al.*, 2012). Even thou there are undesirable compounds present including ANF such as trypsin inhibitors, lectins, flatulence-producing ROF and well as being hard to digest (Marero *et al.*,1998; Kokiladevi *et al.*,2005). A way to eliminate the majority of these effects is by fermenting soybeans with *L. plantarum* to produce different foods that have similar compositions. Lactic fermentation provides higher mineral, vitamin content, as well as reduced trypsin inhibitors, tannin and phytate and higher protein and nutritional content. The positive compounds in soybeans are also more easily digested and absorbed following fermentation. It can however be used as

supplements in cereals for making infant food. Raffinose metabolism and utilization by *L. plantarum* can be employed in the preparation of a good weaning diet for nutritional improvement in children.

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1.0 APPENDICES

1.1 Preparation of Media

LAB was isolated using MRS (De man Rogosa and Sharpe) medium both agar and broth. 52g of prepared and compounded samples were dissolved and homogenized in a water bath and the pH adjusted to 5.5, it was sterilized at 120^0C for 15mins. It was then allowed to cool to 45^0C before pouring into plates (De man Rogosa and Sharpe, 1960).

1.2 Preparation of Standard Curve for Reducing Sugar Determination

Standard maltose concentrations were prepared within the range of 0.2mg/ml - 3.0mg/ml maltose into the requisite medium. This was treated as above discussed above. (Appendix). The result was then used to construct a standard curve. The spectrophotometric values were then extrapolated as maltose equivalent from the standard curve (Bernfeld, 1955).

1.3 Protein Determination

The modified Lowry *et al* (1951) method of protein estimation was used for this determination. To 0.1ml of the cell-free supernatant (enzyme) was added Reagent A which is a mixture of 2g anhydrous Na_2CO_3 dissolved in 100ml of 0.1N NaOH. 1% Sodium tartarate was also prepared and 0.25g of Cupric Sulphate was dissolved in 50ml of the prepared 1% Sodium tartarate which represent Reagent B. Reagent C was prepared by adding 50ml of Reagent A to 1ml of Reagent B. Reagent B and C were prepared only when ready to use while Reagent D is the Diluted Folin and Cicalteau Reagent. 0.1ml of the cell-free supernatant was dispensed into a test tube, 0.5ml of reagent grade water was added to it and 3.0ml of Reagent C was added to the mixture after which 0.3ml of Reagent D was added. This was mixed rapidly after adding Reagent D. it was then left at room temperature for 30 mins. The optical density was determined using Cecil ce 2031 automatic spectrophotometer at 670nm. A blank was prepared with the supernatant and was used to set the spectrophotometer at zero (Lowry *et al.*, 1951).

1.4 Construction of Standard Curve for Protein Determination

1g of casein was measured and dissolved in 100ml of distilled water. This gives 10mg/ml. This was then serially diluted to give 1mg/ml; 0.1mg/ml; 0.01mg/ml and 0.001mg/ml. The absorbance was read at 670nm. This was then used to construct the standard curve by plotting A_{670nm} against protein content (Lowry *et al.*, 1951).

1.5 Preparation of Standard Curve for Oligosaccharide Determination

2g of HPLC grade raffinose was measured and dissolved in 100ml of distilled water. This gives 10mg/ml. This was then serially diluted to give 1mg/ml; 0.1mg/ml; 0.01mg/ml and 0.001mg/ml. The flame photometry was read at 270nm according to the standard for complex sugars determination. This was then used to construct the standard curve by plotting A_{270nm} against sugar content (Black *et al.*, 2007).

1.6 Standard of Indophenol Solution for Vitamin C Determination

0.05 g 2:6 Dichlorophenol Indophenol was dissolved in water. This was diluted to 100 ml and standardised. 0.0500 g pure ascorbic acid was dissolved in 600 ml of 20% meta- phosphoric acid and diluted with water to exactly 250 ml. 10 ml of this solution was pipetted into small flask and titrated with the Indophenol solution until a faint pink colour persisted for fifteen seconds. The concentration was expressed as milligrams per ascorbic acid equivalent to 1.0 ml of the dye solution.

The dye solution keeps for a few weeks if stored in the refrigerator, but it should be standardised before use against a freshly prepared solution of ascorbic acid.

1.7 Preparation of Standard Curve of Riboflavin

From the stock solution of standard Riboflavin, prepare 0.1- 0.5 ppm solution. Then take the absorbance on a spectrophotometer, use this to plot the standard curve. Then extrapolate the result from the readings on the standard curve.

1.8 Preparation of Standard Curve for Thiamine

This is prepared from Tannic Acid. Prepare 0-10 ppm of the Tannic Acid and treat as the sample. Then take the absorbance on a spectrophotometer. Use this to construct the standard curve for Thiamine.

1.9 Estimation of Standard Curve for Niacin

Prepare a blank containing all the reagents used in the extraction. Also prepare standard solutions of the reference Niacin at 1 to 10 ppm and add 1ml cyanogen bromide per ml to them. Measure the absorbance at 650nm. Use the Absorbance to plot a standard graph for the determination. Extrapolate from the reading of absorbance of the sample from the graph to find the concentration of Niacin in the unknown sample.

2.0 Raffinose standard curve

Pure raffinose (Sigma) was prepared in concentrations of 0, 1, 2, 3, 4 and 5 mg/ml. It was then measured in a UV/VIS spectrophotometer (uniscope 20) at a wavelength of 550. The values obtained were used to construct a standard curve (Muzquiz *et al.*, 1992).

2.1 MRS (De Man Rogosa and Sharpe) Medium (composition)

Reptone (Oxoid)	5.0g
Lab-lemco powder (oxoid) Meat Extract	4.0g
Yeast Extract (oxoid)	2.0g
Dextrose	10.0g
Tween 80	0.5ml
Di-potassium hydrogen phosphate	1.0g
Sodium acetate	2.5g
Tri-ammonium citrate	1.0g
Magnesium sulphate	0.1g
Mangnese sulphate	0.025g
Agar (oxiod)	7.5g
Distilled Water	500ml

b) MRS Medium (for fermentation studies of LAB)

Constituent is the same as above except that glucose and meat extract are normally omitted. Adjust the pH to 5.5 – 6.5. Add 0.004% bromocresol as indicator. 10% solution of the test substrates are prepared and sterilized by filtration. The required substrates are added aseptically to give a final concentration of 2%.

2.2 Glucose Phosphate Medium

Peptone	-	5.0
Glucose	-	5.0
Dipotassium hydrogen phosphate	-	1.0
pH	-	7.5
Distilled Water	-	1 litre

Arginine MRS Broth

Medium to test for the produce of NH_3 from Arginine by LAB. MRS Broth + 0.3% (w/v) I-Arginine monohydrochloride

2.3 Nitrate Reduction Test

Nitrate Peptone H_20 (for detecting nitrate reducing ability LAB)

Potassium Nitrate (AR grade)	0.2g
Peptone Water	1litre

Peptone H_20 (suitable for Indole Tests)

Tryptone	10.0g
NaCl	5.0g
Distilled Water	1 litre

2.4 Medium for Homofermentative /Heterofermentative Test

Yeast Extract	2.5g/ litre
Glucose	50.0g/litre
$MnSO_4.7H_20$	1000ml

Reconstituted skimmed milk with 80g/80ml distilled water

Nutrient agar 5.6g/19ml distilled H_20

Lead acetate papers (to test for H_2S production)

Soak 5cm x 5cm strips of filter paper in saturated lead acetate solution and heat at 50 – 70^oC until dry

2.5 Methyl Red Solution (for the methyl test)

Methyl red	-	0.1g
Ethanol 97%	-	300ml
Distilled H_20	-	500ml

Dissolve the methyl red in the ethanol and make up to 500ml with distilled water

2.6 Kovac's indole Reagent (to test for indole production in tryptone water)

Amyl or Isoamyl alcohol	-	150 ml
D-Dimethylaminobenzaldehyde	-	10g
Conc. Hcl acid	-	50ml

c) Barritt's Method of Voges Proskaeur Reaction

Add 3ml of 5% alcoholic alpha – naphthol solution and 3ml of 40% KOH solution to the broth that has been incubated before.

2.7 Simmons Citrate Medium

Magnesium sulphate	0.2g
Monoammonium phosphate	1g
Dipotassium phosphate	1g
Sodium citrate	2g
Sodium chloride	5g
Agar	15g
Bromothymol Blue	0.08g

Suspend 24.2g of simmons citrate agar in 1000ml of cold distilled water and heat to boiling to dissolve the medium completely. Distribute in tubes or flasks and sterilize at 121^{o}C for 15 minutes

2.8 Reagent and Dyes

Grams's Crystal Violet

Solution A	g/litre
Crystal violet	10.0
Ethyl Alcohol (95%)	100ml

Solution B

Ammonium Oxalate	1.0
Distilled water	100ml

Solution A and B were mixed with together to give crystal violet

Solution C

Safranin O	0.25g
Ethyl alcohol	10.0ml
Distilled water	100.0ml

2.9 Gram's iodine

Potassium iodide	2.0g
Iodine	1.0g
Distilled water	100ml

3.0 1% oxidase reagent

Tetramethyl-p-phenylenediamine hydrochloride	0.1g
Distilled water	10ml

3.1 DNSA Reagent

3,5 Dinitrosalicylic Acid	1g
0.1 N NaoH	20ml
Potassium Sodium Tartarate	20g
Distilled water	100ml

Amylase Assay

1.2% soluble starch

Soluble starch	1.2g
Distilled water	100ml

$$\text{Molarity} = \frac{\text{Conc. (g/l)}}{\text{Molar masses}}$$

$$\text{Normality} = \frac{\text{conc. (g/l)}}{\text{Eq. wt}} \quad \text{Eq. wt} = \frac{\text{molar mass}}{\text{valency}}$$

3.2 Protein Estimation Reagent by Lowry et al; 1951

Reagent A

Anhydrous Na_2CO_3	2g
0.1 N NaOH	100ml

Reagent B

1% Sodium tartarate	
Sodium tartarate	1g
Distilled water	100ml
Cupric sulphate	0.25g
Reagent B (prepare when ready to use)	
Sodium tartarate	50ml
Cupric sulphate	0.25g

Reagent C (prepare when ready to use)

Reagent A	mix	50ml
Reagent B		1ml

Reagent D Diluted Folin and cicalteau Reagent (standard) for the experiment

Dilute Folin and cicalteau Reagent	0.3ml
Enzyme (filterate from Broth culture)	1ml

3.3 Enzyme Assay Method According To Bernfeld, 1955

1ml of the enzyme filtrate was added to 1ml of the standard starch substrate (1.2% soluble starch in 0.1m phosphate buffer at pH 6.0). This was incubated at 30^0C for 5 minutes. The reducing sugar produced was determined by adding 1ml of DNSA reagent. This was heated in boiling water bath at 100^0C for 5 minutes and cooled with tap water after which 20ml distilled water was added. The absorbance of the resultant solute was determined at 540nm with the aid of Cecil 2031 spectrophotometer. 1ml of the inoculated medium similarly treated was used to set the transmittance at zero. The amount of sugar produced was determined from a standard curve obtained by recording the absorbance of increasing concentration of aqueous solution of maltose ranging from 0.2mg/ml – 2.0mg/ml. The result was then expressed as amylase unit (Bernfeld, 1955).

3.4 Determination of Reducing Sugar

1ml of the enzyme filtrate was added to 1ml of the standard starch substrate (1.2% soluble starch in 0.1m phosphate buffer at pH 6.0). This was incubated at 30^0C for 5 minutes. The reducing sugar produced was determined by adding 2ml of DNSA reagent. This was heated in boiling water bath at 100^0C for 5 minutes and cooled with tap water after which 20ml distilled water was added. The absorbance of the resultant solute was determined at 540nm with the aid of Cecil 2031 spectrophotometer. 1ml of the inoculated medium similarly treated was used to set the transmittance at zero. The amount of sugar produced was determined from a standard curve obtained by recording the absorbance of increasing concentration of aqueous solution of maltose ranging from 0.2mg/ml – 2.0mg/ml. The result was then expressed as amylase unit (Bernfeld, 1955).

3.5 Procedure for Raffinose, Stachyose and Verbascose

Wavelength: 340 nm

Cuvette: 1 cm light path (glass or plastic)

Temperature: optimally 40°C in a dry hot-block heater or in the spectrophotometer, but otherwise ~ 25°C

Final volume: 2.62 mL

Sample solution: 3-250 µg of raffinose per cuvette

(in 0.10-0.20 mL sample volume)

Calculation:

The absorbance differences (A2-A1) for blanks and samples was determined. The absorbance difference of the blank from the absorbance difference of the corresponding sample as follows was subtracted:

$\Delta A = (A2\text{-}A1)_{sample} - (A2\text{-}A1)_{blank}$

Determination of Oligossaccharide

The concentration of the oligossacharides was calculated as follows:

$c = V \times MW \times \Delta A$ [g/L]

where:

V = final volume [mL]

MW = molecular weight of the substance assayed [g/mol]

ε = extinction coefficient of NADH at 340 nm

= 6300 [l x mol-1 x cm-1]

d = light path [cm]

v = sample volume [mL]

for raffinose:

c = 2.62 x 504.5 x Δ A raffinose [g/L]

6300 x 1 x 0.2

= 1.049 x ΔA raffinose [g/L]

For verbascose

c = 2.62 x 624.59 x Δ A raffinose [g/L]

6300 x 1 x 0.2

= 1.299 x ΔA raffinose [g/L]

For starchyose

c = 2.62 x 666.574 x Δ A raffinose [g/100g]

6300 x 1 x 0.2

= 1.386 x ΔA raffinose [g/L]

Note: When analysing solid and semi-solid samples which are weighed out for sample preparation, the content (g/100 g) is calculated from the amount weighed. **(**Saini, & Knights, (1984); Beutler, (1988).

3.6 Determination of Glucose, Sucrose and Fructose

Procedure:

Wavelength: 340 nm

Cuvette: 1 cm light path (glass or plastic)

Temperature: ~ 25°C

Final volume: 2.42 mL (D-glucose)

2.44 mL (D-fructose)

Sample solution: 4-80 µg of sucrose + D-glucose + D-fructose

per cuvette (in 0.10-1.00 mL sample volume)

Read against air (without a cuvette in the light path) or against water

Pipette into Blank Sucrose /Blank D-Glucose/Blank D-fructose

Prepare standard graphs for the sugars from 4 to 80 µg .

Calculation:

The absorbance differences (A2-A1) and (A3-A2) for both blanks and samples were determined, and the values of ΔAD-glucose, ΔAsucrose and ΔAD-fructose was calculated as described below:

Determination of free D-glucose:

ΔAD-glucose = (A2-A1) sample - (A2-A1) blank (from the D-glucose/ D-fructose sample).

Determination of sucrose:

The difference between ΔAtotal D-glucose and ΔAD-glucose (from the D-glucose/D-fructose sample) yields **ΔAsucrose**.

Determination of free D-fructose:

The absorbance difference (A3-A2) for both blank and sample (D-glucose/D-fructose sample only was determined. The absorbance difference of the blank was subtracted from the absorbance difference of the sample thereby obtaining **ΔAfructose**.

The values of ΔAD-glucose, ΔAsucrose and ΔAD-fructose should be at least 0.100 absorbance units to achieve sufficiently accurate results.

The concentration of D-glucose, sucrose and D-fructose can be calculated as :

$$c = \frac{V \times MW \times \Delta A}{\varepsilon \times d \times v} \text{ [g/L]}$$

***Where*:**

V = final volume [mL]

MW = molecular weight of the substance assayed [g/mol]

ε = extinction coefficient of NADPH at 340 nm

= 6300 [l x mol-1 x cm-1]

d = light path [cm]

v = sample volume [mL]

ΔAtotal D-glucose = (A2-A1) sample - (A2-A1) blank

It follows for D-glucose:

c = 2.42 x 180.16 x ΔAD-glucose [g/L]

6300 x 1 x 0.1

= 0.6920 x ΔAD-glucose [g/L]

for sucrose:

c = 2.42 x 342.3 x ΔAD-sucrose [g/L]

6300 x 1 x 0.1

= 1.315 x ΔAsucrose [g/L]

f*or D-fructose:*

c = 2.44 x 180.16 x ΔAD-fructose [g/L]

6300 x 1 x 0.1

= 0.6978 x ΔAD-fructose [g/L]

Samples diluted during preparation, the result must be multiplied by the dilution factor, F.For solid and semi-solid samples weighed out for sample preparation, the content (g/100 g) was calculated from the amount weighed.

(Beutler, (1988); Outlaw and Mitchell, (1988).

3.7 TE buffer (10ml Tris – Hcl, 1mM EDTA, pH 7.5)

3.8 TAE buffer (4.84g Tris base, 1.14g glacial acetic acid, 2ml 0.5M EDTA, pH