PRODUCTION OF PROBIOTIC FRUIT JUICE (ORANGE AND WATERMELON) USING LACTIC ACID BACTERIA ISOLATED FROM FRESH COW MILK

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(MTech/SLS/2017/7483)

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ABSTRACT

Probiotication of fruit juice by *Lactobacillus* sp is an essential component of the human diets in the food industry. This study is focused on the production of probiotic fruit juice from orange and watermelon. Probiotic viability, physicochemical (titratable acidity, total soluble solid, pH and reducing sugar), antimicrobial activity and sensory evaluation of the fermented and stored probiotic orange and watermelon juice using lactic acid bacteria (LAB) (*Lactobacillus plantarum* strain MerLAB1and *Lactobacillus paracasei* strain MerLAB2) as single and mixed (consortium) starter was investigated. Results revealed that there was an increase in the number of lactic acid bacterial count (0.09 - 3.37 $\times 10^6$ CFU/mL), titratable acidic % (0.030-0.050) and reduction in pH (4.28-3.97), reducing sugar ^oB (4.49-3.49) and total soluble solid (4.12-3.81) during fermentation. *Lactobacillus plantarum* and *Lactobacillus paracasei* mixed culture (T_3) probiotic juice samples had the best antagonistic activity against the pathogenic test organism (*Escherichia coli*) with an inhibition zone of 30mm after 72 hours of fermentation. The probiotic LAB were viable throughout the storage time of 4weeks at 4 ^oC with the cells of $1.00\pm0.00\ 10\times10^9$ CFU/mL in the juice sample. There was no significant difference at $P \geq 0.05$ in terms of sedimentation, aroma, colour, or appearance during the time of storage. The orange and watermelon juice supported the viability, lactic acid production, and the antagonistic potential of the probiotic candidate. This study suggests that the production of fruit juice supplemented with *Lactobacillus plantarum* strain MerLAB1and *Lactobacillus paracasei* strain MerLAB2 is essential in the food industry.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the Study

Foods have many roles such as satisfying hunger, providing necessary nutrients, improving health, promoting a state of physical and mental well-being as well as preventing or reducing nutrition-related diseases (Patel, 2017). However, consumer's awareness towards the association between food and health has flare- up interest in "healthy foods" in recent

years (Shah and Prajapati 2013). In addition to the traditional nutritional effects, “functional foods” exert beneficial health effects on body. Well-recognized examples of functional foods are those containing bioactive compounds like dietary fibers, oligosaccharides, vitamins, minerals and active “friendly” bacteria, called probiotics that promote the equilibrium of intestinal microflora (Jankovic *et al*., 2010; Shah and Prajapati, 2013). The functional foods market is growing globally and represents one of the most fascinating areas of investigation and innovation in the food sector as suggested by the increasing number of scientific literatures (Patel, 2017).

Probiotics are live microbial feed supplements which enhance the health of consumer by improving the balance of micro flora in the gut when ingested live in sufficient numbers (Tayo and Akpeji, 2016). The consumption and development of probiotic foods is increasing by the day due to the consumer awareness about functional foods of which has ability to maintain good health. The role of probiotic organisms as complementary therapy in combating large number of gastrointestinal disorders and their ability to enhance immune response attracts global attention. Probiotic provide several health benefits, including antimicrobial, anti-tumor, anti-cholesterol, immuno-modulation, anti-diabetic, treatment of diarrhea and lactose intolerance (Hossain *et al.*, 2020).

Consumer demand for non-dairy based probiotic products has increased due to the problems of lactose intolerance and cholesterol content associated with the consumption of fermented dairy products. In this respect, fruit juice offers an alternative to produce probiotic foods using *Lactobacillus* species as some *Lactobacillus* species were found to have the capacity to reduce blood cholesterol levels (Hossain *et al.*, 2020).

Lactic acid bacteria (LAB) are common inhabitants of fermented products (milk, meat and vegetables) and gastrointestinal tracts, most of which are responsible for maintaining a balance of the micro-biota of healthy host. This group of bacteria has the ability to colonize the gastrointestinal tract and hence ferment carbohydrate which produces lactic acid as the major metabolic end product that aids digestion. Also as part of its host benefits, it prevents the action of pathogenic microorganism through the production of inhibitory substances (metabolites) and a formation of a bio-film to protect the intestinal mucosal membrane (Vantsawa *et al.*, 2017). LAB metabolizes different substrates leading to biochemical changes in composition. Lactic acid fermentation is recognized to improve nutritional properties, flavor, and health-related aspects of food products (Garcia *et al.*, 2020). This process improves the organoleptic properties of food matrixes, their acceptability and increases the shelf-life properties (Filannino *et al*., 2013).

Fruit juices have been suggested as an ideal substrate for the development of non-dairy probiotic beverages. In addition to the healthy ingredients of fruit juices, such as, vitamins, antioxidants and polyphenols, they also offer several advantages for the growth and survival of probiotic microorganisms. More specifically they contain high content of nutrients and sugars which are important for the growth of probiotic (Vijaya *et al*., 2015). Fruit juices have taste profiles that are pleasant and are easily acceptable from all age groups. All these characteristics have attracted the interest of several researchers for the development of probiotic beverages based on fruit juices (Kandylis *et al.*, 2016).

The development of new lactic acid fermented fruit juices has recently generated interest. These products have been shown to have considerable market value because of the combination of nutritional advantages of the raw materials and benefits resulting from

lactic fermentation. Some attempts to develop lactic fermented fruit or vegetable juices have been made on various substrates. However, most studies were performed with *Lactobacillus* spp., and some with *Bifidobacterium* spp., *Leuconostoc* spp. and *Weissella* spp (Nguyen *et al.*, 2019). To be successful, the manufacturing process of these juices requires a mild Pasteur and inoculation with selected starters. Pasteurization reduces microbial population in the juice, possibly inactivates foodborne pathogens and hence favors the implantation of the starter (Shet *et al*., 2017). To obtain a well-accepted juice, the sensory characteristics have to be carefully examined by adjustment of the mix of fruit or vegetables, to avoid an unpleasant acidic or astringent beverage and by the choice of starters to avoid undesirable compounds, flavors or biogenic amines. The ability of starter strains to grow in the juice is an important parameter to consider for successful fermentation or to expect some probiotic effects (Garcia *et al*., 2020). Long-term survival of bacteria in the fermented juice and putative probiotic properties of starters in the juice are regarded as advantages (Garcia *et al.*, 2020).

Fruit juices offer several advantages as probiotic vehicles, they are rich source of nutrients (antioxidants, minerals, and vitamins) and their natural sugars contribute to the probiotics growth. Moreover, the fruit juices also have a good refreshing taste and are a consumption choice for people of all age groups. Another advantage is that for these juices digestion in the stomach is faster than that for dairy products. Thus, the microorganism spends much less time in the acidic environment of the stomach (Ding and Shah, 2008). Several studies using fruit juice for probiotic drinks production were recently published. Pineapple, cantaloupe melon, cashew apple, apple, orange, black currant, banana, and blueberry are

some of the fruit juices used as food matrices for probiotic bacteria delivery (Costa *et al*., 2013; Pereira *et al*., 2011).

Fruit juices are essential components of the human diet. Apart from being good sources of vitamins, minerals, and fiber, these foods are also rich source of potentially bioactive compounds (Palafox-Carlos, *et al*., 2011).

1.2 Statement of Research Problem

Most of the presently available probiotics foods are based on milk but there are several problems associated with consumption of milk such as lactose intolerance and increase in the level of the cholesterol of the consumers. Lactose intolerance and the cholesterol content are two drawbacks related to the consumption of milk and milk products (Patel, 2017).

Currently, products are usually marketed in the form of fermented milk and yoghurt. It has also been suggested that fruit juice could serve as a good medium for cultivating probiotics. The use of probiotics in the fruit juice industry offer to consumers with special needs (vegetarian people with allergic reactions to milk proteins).

1.3 Justification for the Study

One of the most promising areas of development in the human nutritional field over the last two decades has been the use of probiotics and recognition of their role in human health and disease. Lactic acid-producing bacteria are the most commonly used probiotics in foods. It is well known that probiotics have a number of beneficial health effects in humans and animals. They play an important role in the protection of the host against harmful microorganisms and also strengthen the immune system. Some probiotics have also been

found to improve food digestibility and reduce metabolic disorders. They are safe, acid and bile tolerant, and able to adhere and colonize the human intestinal tract (Zielińska *et al*., 2018). Fruit juices represent a suitable carrier for the delivery of probiotics. Since, fruits are naturally rich in essential macro- and micro-elements, incorporation of probiotics into fruit juices makes them healthier (Patel, 2017).

Many foodstuffs containing probiotics are of milk origin but the interest of both manufacturers and consumers is extended also to non-dairy products. Greater attention is now given to fruit juices that are considered to be healthy due to their high content of bioactive compounds and are consumed by all age groups of the population. Fruit juices with added probiotics can be a choice for people who cannot consume dairy products for health (lactose intolerance) (Antunes *et al*. 2013; Martins *et al*. 2013).

1.4 Aim and Objectives of the Study

1.4.1 Aim

The aim of this study was to produce probiotic fruit juice (orange and watermelon) using lactic acid bacteria isolated from fresh cow milk.

1.4.2 Objectives

The objectives of the study were to:

i. isolate and identify lactic acid bacteria from fresh cow milk.
ii. produce water melon and orange probiotic juices using lactic acid fermentation

iii. determine physico-chemical properties of the probiotic juices during and after fermentation.

iv. determine the cell viability, microbiological quality, antagonistic effect on a test organism and physico-chemical parameters of the probiotic fruit juices during storage.

v. evaluate the sensory properties of the probiotic fruit juice and shelf life stability.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Food Fermentation

Food fermentation helps in stabilization and transformation of food materials via metabolic activity of microorganisms especially in different type of perishable foods such milk, vegetable or fruits or any other water rich foods (Swain *et al.*, 2014). It is also applied as food preservation tool for creating desirable organoleptic, nutritional and functional attributes in fermented food with significant contribution in diet of developing countries. There is still found a renewed interest in fermented food products due to purported health benefits for metabolic syndromes such as obesity, various food allergies and intolerances (lactose intolerance and gluten intolerance) and lifestyle choices (vegetarianism and veganism). Natural fermented food has shown the increased interest by consumers in everything perceived natural and promote health and longevity (Srivastava, 2018). In fermentation process, biochemical reactions are involved during lactic acid fermentation to yield organic acids, alcohols, aldehydes and ketones. Temperature, water activity, hydrogen ion concentration (pH), oxygen availability and substrate are main parameters that influence the food fermentation process (Srivastava, 2018).

2.1.1 Food fermentation principle

Fermented food is the precious wealth given by nature to mankind; the preparation of fermented food involves many microorganisms Generally Regarded as Safe (GRAS) and edible directly (Tiwari *et al*., 2012). Lactic acid bacteria are one of the most important microorganisms in food production. Different countries and regions have their own

characteristics of traditional lactic acid fermented food based on lactic acid fermentation principle. Lactic acid bacteria as the main fermentation agent through lactic acid fermentation to form short-chain fatty acids acidification and accompanying role of biological metabolism improves the sensory characteristics of food matrix profile. The nutritional value of fermented products was improved by releasing amino acids and forming vitamins. The lactic acid bacteria bio-antagonism substances such as short- chain fatty acids, bacteriocins and hydrogen peroxide which have significantly improved the preservation and safety of the products and are typical models for the natural green processing of foods (Landete, 2017). The role of food in developing human health and wellbeing has been known since the times of Hippocrates, whose saying, “Let food be thy medicine and medicine be thy food” frequently repeated today has become the slogan of supporters of “treating” with food (Zielińska *et al.*, 2018). Therefore, the introduction of beneficial bacterial species to Gastro intestinal tract may be a very attractive option to re-establish the microbial equilibrium and prevent diseases (Tiwari *et al*., 2012).

2.2 Fruits

Fruits have been reported as important source of nutrients in many parts of the world and offer many advantages over dietary supplements because of its low cost and wide availability Science and Owerri (2015) have reported that fruit and vegetables play a significant role in human nutrition, especially as sources of dietary fibre, minerals, vitamin C (ascorbic acid), vitamin A, thiamin (B_1), niacin (B_3), pyridoxine (B_6), folic acid, vitamin E. Hence the reason fruits and vegetables are also called protective foods.

Some components of fruits and vegetables like fibers, mineral and vitamins especially phytochemicals and antioxidants, are strongly associated with reduced risk of cancers, heart diseases, stroke and other chronic diseases. Thakur (2015) have reported that consumers are encouraged to eat up to 10 fruits and vegetables per day in some countries due to the highest antioxidant capacity of them. The industrial potential of many fruits and vegetables available in Nigeria is enormous. We need to embark on massive production of these fruits and vegetables not only for their high nutritive value but for enhancing the establishment of many processing industries. The development of their industrial uses will stimulate large scale production of the crops and enhanced diversification of entrepreneur to site processing plants in the rural areas which will improve the quality of life of the rural population and reduce the rate of rural-urban migration.

2.2.1 Watermelon

Watermelon (*Citrullu*s spp.) is a xerophytic tropical fruit, belonging to a *Cucurbitaceae* family. Lycopene, Vitamins A, B_6, C, carotenoids, and antioxidants are some nutrients found in watermelon (Maoto *et al*., 2019). Watermelon consumption has increased owing to its nutritional profile and allied health benefits. The fruit is effective in reducing cancer, cardiovascular disorders, diabetes, blood pressure and obesity (Dube *et al*., 2020).

2.2.1.1 *Health benefits of watermelon*

Watermelon is a diuretic and contains large amounts of β-carotene, a precursor of Vitamin A (Leskovar*et al.*, 2004; Naz *et al.*, 2014). Lycopene, a phyto-chemical, is an important intermediate in the biosynthesis of many carotenoids, including β-carotene, responsible for yellow, orange, or red pigmentation. Like all carotenoids, lycopene is a polyunsaturated

hydrocarbon and insoluble in water. Lycopene deep red color is responsible for its antioxidant activity and has been extensively studied. The fruit is effective in reducing cancer, cardiovascular disorders, diabetes, blood pressure, and obesity (Lum *et al.*, 2019; Naz *et al.*, 2014). Quantitative assessment indicates that watermelon has 46 % calories, 20 % vitamin C, and 17 % vitamin A and has higher lycopene than tomato (Biswas *et al.*, 2017). Watermelon is inexpensive, nutritious and readily available to all socio-economic groups in Africa throughout the summer. It is a common fruit in Africa with the potential to improve nutrition, boost food security, foster rural development and support sustainable land conservation (Ufoegbune *et al.*, 2014).

2.2.2 Orange

Citrus sinensis or sweet orange originated from South East Asia, but is consumed all over the world as an excellent source of vitamin C (Shravan *et al.*, 2018). It is a powerful natural antioxidant that builds the body immune system. Important phytochemicals like liminoids, synephrine, hesperidin flavonoid, polyphenols, pectin, and sufficient amount of folacin, calcium, potassium, thiamine, niacin and magnesium are also present. These biologically active compounds prevent arteriosclerosis, cancer, kidney stones, stomach ulcers and reduction in cholesterol level and high blood which promote human health (Etebu *et al.*, 2014). Oranges, like other citrus fruits, is an excellent source of vitamin C that provide human with vitamin C as its major nutrient which is a powerful natural antioxidant. The ascorbic acid content represents a stimulating factor for citrus fruit consumption (Aderinola, 2015).

Oranges form a rich source of vitamin C, flavonoids, phenolic compounds and pectins, one orange provides 116 percent of the daily requirement for vitamin C. Vitamin C is the primary water- soluble antioxidant, which prevents free radical generation in the body and damage to the tissues in the aqueous environment both inside and outside cells (Fruits and Olanaceae, 2011).

2.2.2.1 *Health benefits of orange*

Orange contain very good amount of vitamin A, and other flavonoid antioxidants such as alpha and beta carotenes, beta-cryptoxanthin, zeaxanthin and lutein, compounds that have antioxidant properties. Vitamin A is necessary for maintaining healthy mucus membranes, skin and essential for vision. It is also a very good source of B- complex vitamins such as thiamin, pyridoxine and folates. These vitamins are essential in the sense that body requires them from external sources to replenish. Orange fruit also contains a very good amount of minerals like potassium and calcium. Potassium is an important component of cell and body fluids, it helps to control heart rate and blood pressure. Vitamin A is also required for maintaining healthy mucus membranes and skin and is also essential for vision. Consumption of natural fruits rich in flavonoids helps body to protect from lung and oral cervical cancers. Orange fruit also contains a very good amount of minerals like potassium and calcium. Potassium is an important component of cell and body fluids and helps to control heart rate and blood pressure, the alkaline properties in the orange stimulate the digestive juices, thus, reliving constipation. Regular intake of orange juice reduces the chances in the formation calcium oxalate which causes kidney stones. Polyphenols present in oranges prevents viral infections. Oranges protect the skin from damage caused by free

radicals, thereby helping to look young and keeps the skin fresh and glowing (Tsuda *et al*., 2004).

Oranges can be processed into juice, which can be consumed directly or further processed into concentrate (Etebu *et al*., 2014).

2.2.3 Fruit juices as a matrix for probiotic microorganism's delivery

Fermented dairy products are good food matrices for probiotics delivery. However, the consumption of these products is limited due to a large number of individuals who are lactose intolerant or are on cholesterol-restricted diets (Martins *et al*., 2013). Therefore the non dairy probiotic products, including food matrices based on fruit have been widely studied (Fernandes Pereira and Rodrigues, 2018). Fruits are an appropriate matrix for the growth of probiotics, the survival of these microorganisms in such a matrix is even more complex than in dairy products, because the lactic acid bacteria need to protect themselves from the acid conditions of the fruit (Shah, 2007). It is important to acquire new knowledge on the different operations of the fruit juices production to identify the influence of the processing parameters and steps involved in the survival of probiotic microorganisms (Cruz *et al*., 2009). There are two ways of turning a fruit juice into a probiotic food, the microorganism addition to the fruit juice and the fermentation with probiotic microorganisms. The first attempt at making probiotic juices was the addition of *Lactobacillus* in fruit juices. This technique is successful if the strain is acid tolerant. Fermentation presents some advantages over the addition because the microbial strain growth into the juice results in a low-sugar product and a more adapted microbial strain, which might contribute to higher survival rates. Another advantage of fermentation is the

production of metabolites that can help to increase the product quality, such as bacteriocins, which avoids microbial contamination during storage (Fernandes and Rodrigues, 2018).

Fonteles *et al*. (2012) evaluating the optimum conditions for probiotic cantaloupe melon juice, observed that an initial pH of 6.1 resulted in good cell viability (8.3 CFU/mL) at the end of the fermentation, indicating that this juice is a suitable vehicle for *Lactobacillus casei* delivery. Nagpal *et al.* (2012), using two *Lactobacillus* strains (*Lactobacillus plantarum* and *Lactobacillus acidophilus)* for the production of probiotic orange juice and probiotic grape juice, noted that the two cultures were able to maintain good viability in both juices, despite their high acidity. The survival of the strains during storage of the probiotic food product is imperative to ensure their health benefits. Among the factors that affect the probiotic bacteria viability are the microbial strain, the fruit juice composition (acidity, carbohydrate content, nitrogen sources, mineral con- tent), and the possible interactions of the probiotics strains with the food matrix components (Ranadheera *et al*., 2010). Some studies showed that a high fiber and protein content is favorable for maintaining the viability of probiotic bacteria during refrigerated storage of fruit juices such as orange, apple, grapefruit, black currant, pineapple, and lemon (Champagne and Gardner, 2008; Ding and Shah, 2008; Fernandes and Rodrigues, 2018)

Pereira *et al*. (2011), studied the fermentation of cashew apple juice, a very popular juice in Brazil, and found a high viability of the *L. casei* during the refrigerated storage for 42 days, with viable cell counts higher than 8.00 log CFU/mL. The use of specific nutrients in the fruit juices can influence the survival of the lactic acid bacteria. Shah *et al.* (2010) evaluated the survival of three strains of bacteria: *L. rhamnosus*, *Bifidobacterium lactis*, and *L. casei* in fruit juices enriched with grape seed extract, green tea extract and vitamin

C. After 42 days of storage, the product that had an initial concentration of 8.32 CFU/mL, presented cell viability reduced to 4.29, 7.41 and 6.44 log CFU/mL, respectively.

Mousavi *et al.* (2011) reported that *L. plantarum* and *Lactobacillus delbrueckii* showed optimal probiotic growth and maintained their viability during 14 days of storage at 4 ^{o}C in the fermented pomegranate juice, while *L. acidophilus* and *L. paracasei* lost their viability under the same conditions. Moreover, the authors observed that the citric acid (the main organic acid present in pomegranate juice) was rapidly consumed by all the probiotic microorganisms.

2.2.4 Fruit juice fermentation by lactic acid bacteria.

Lactic acid bacteria (LAB) are extensively used in food fermentation. They are use improve to the organoleptic characteristics and nutritional values of fermented products. During the fermentation, LAB transforms indigestible substances into others easier to digest and produce different antimicrobial compounds. Some of these bacteria are called "probiotic" and are known to have health-promoting attributes. Different researchers studied the suitability of fruit and vegetable juices for the development of probiotic beverages. Mousavi *et al.* (2011) reported that the fermented beverages consumption increases the total number of LAB in the intestinal tract, which helps in enhancing immunity against common pathogens. Among all LAB, *Lactobacillus plantarum* is the most used species for vegetable fermentation. LAB use carbon sources and free amino acids present in the medium to produce metabolites of interest. Lactic acid is the major organic acid produced by LAB, and it has a role in the reduction of the pro inflammatory cytokine secretion of Toll-like receptor- (TLR-) activated, bone marrow-derived macro-

phages and dendritic cells in a dose-dependent manner. Besides organic acids production many secondary metabolites are synthesized such as bioactive peptides, fatty acids, exopolysaccharides and vitamins. LAB increases also the antioxidant activity of fermented products thanks to enzymes' activity such as β-glucosidase and esterase. Moreover, they produced phenol derivatives are usually a source of the end products' aroma, Several juice substrates are fermented using LAB (Ayed *et al*., 2020).

2.3 Lactic Acid Bacteria (LAB)

LAB have traditionally been associated with food and food fermentations, and are generally considered beneficial micro-organisms, including some strains even as a health promoting (probiotic) bacteria such as *Lactobacillus*, *Streptococcus*, *Enterococcus*, *Lactococcus*, *Bifidobacteria* and *Leuconostoc.* Qian *et al*. (2018) reported that lactic acid bacteria (LAB) are one of the important microorganisms in food fermentation. Lactic acid bacteria are defined as bacteria which produce lactic acid as their major fermentation product. They are Gram positive usually non-motile, non-spore forming, rod and cocci, and catalase negative (Horáčková *et al*., 2018). LAB can only get ATP by fermentation, usually of sugar. Since they do not use oxygen in their energy production, lactic acid bacteria grow under anaerobic condition, but they can also grow in presence of oxygen. They are protected from oxygen by by-products (H_2O_2) because they have peroxidase. That is why these organisms are aerotolerant anaerobes (Zhu *et al*., 2020).

2.3.1 Factors affecting lactic acid fermentation

There are seven factors that influence the growth and activity of Lactic acid bacteria (LAB) in fermenting fruits and vegetables. These are pH, moisture and water activity, O_2

concentration, temperature, nutrients, selected starter culture and inoculum concentration (Montet *et al.*, 2014)

2.3.1.1 *pH*

The pH is a critical factor in preservation and developing aroma and flavor of many fermented fruits and vegetables Most LAB favour conditions with a near neutral pH (Battcock and Azam-Ali, 2001). Certain bacteria are acid tolerant (*Lactobacillus* and *Streptococcus*) and can survive at reduced pH levels 3.0-4.0 (Montet *et al*., 2014)

2.3.1.2 *Oxygen availability*

The oxygen requirements vary from species to species. Unlike many anaerobes, however most LAB is not sensitive to oxygen and can grow in its presence as well as in its absence. They are aero-tolerant anaerobes (Montet *et al.*, 2014).

2.3.1.3 *Temperature*

Temperature is a critical factor for fruit juice fermentation. Most LAB have a temperature optimum between 20 ºC to 30 ºC, there are some (thermopiles) which prefer high temperatures (50-55 ºC) and those with colder temperatures optimal (15-20 ºC) Most LAB work best at temperatures of 18-22 ºC (Ray and Panda 2007).

2.3.1.4 *Salt concentration*

Salting is an important step in vegetable fermentation. Sodium chloride concentration can range from 20 to 80 g/L during fermentation. LAB can tolerate high salt concentrations. This salt tolerance gives them an advantage over less tolerant species and allows lactic acid fermentation that inhibits growth of non-desirable organisms. Salt induces plasmolysis in

plant cells which releases mineral salts and nutrients from the vacuole and creates anaerobic conditions for proper growth of LAB around the submerged product (Wouters *et al.,* 2013).

2.3.1.5 *Water activity*

LAB requires a fairly high water activity (0.9 or higher) to survive. There are a few species, which can tolerate water activities lower than this, but usually, the yeasts and fungi will predominate on foods with a lower activity (Ray and Panda 2007).

2.3.1.6 *Nutrients*

All bacteria require a source of nutrients for metabolism. The fermentative bacteria require carbohydrates, either simple sugars such as glucose and fructose or complex carbohydrates such as starch or cellulose (Wouters *et al*., 2013).

2.3.1.7 *Selected starter cultures*

The selection of starter cultures is based principally on the competitiveness between the starter and the natural flora as well as on the sensory properties of the resulting products (Montet *et al.,* 2014).

2.4 Probiotics

The word probiotic is coined by Kollath (1953) and is derived from the Greek language, which means for life. According to Lilly and Stillwell (1965), probiotics are substances produced by microorganisms that promote the growth of other microorganisms (Food and Agricultural Organization / World Health Organization) (FAO/WHO2001). However, the widely adopted definition states probiotics as live microorganisms which when

administered in adequate amounts confer a health benefit on the host to prevent some diseases or improve health (Vijaya Kumar *et al*., 2015).

Probiotics may be a natural temporary constituent of the resident intestinal microflora, but their population is not always sufficient for therapeutic purposes. The microbiota, the intestinal epithelium, and the mucosal immune system constitute the gastrointestinal ecosystem. All the three components are essential for the complete functional and developmental maturity of the system. The use of antibiotics, immunosuppressive therapy, and irradiation, among other means of treatment, may cause alterations in the composition and have an effect on the gastrointestinal tract flora. Therefore, the introduction of beneficial bacterial species to gastrointestinal tract may be a very attractive option to re-establish the microbial equilibrium and prevent diseases (Tiwari *et al*., 2012).

Probiotics are usually introduced to food and beverages as a component of fermentation process at appropriate stage. Due to their long time survival and multipurpose capacity, there are different rout of administered mechanisms based on age class interval. It can be taken orally in the form of capsule or probiotic food. In order to crate and supply health effect, probiotic cells are constantly viable in the food carries and adapt extreme harsh environment of Gastro intestinal tract (GIT) (Ravinder *et al*., 2012).

2.4.1 Mechanism of action of probiotics

Probiotics have various mechanisms of action currently, three major ways of action of probiotics have been revealed (Abatenh *et al*., 2018). The first one is a competition for nutrients and for ecological niche at this time the indigenous anaerobic flora limits the concentration of potentially pathogenic flora in the digestive tract. Probiotics can have a

direct effect on other microorganisms through inhibition of pathogen adhesion this kind of major defense mechanism is used to maintain internal health condition. *Lactobacilli* and *bifidobacteria* have been shown to inhibit a broad range of pathogens by performing colonization of pathogenic bacteria and finally by doing antagonistic activity against gastrointestinal (Vijaya Kumar *et al*., 2015). This principle in many cases is crucial for the prevention and treatment of infections and restoration of the microbial equilibrium in the gut. The second mechanism is involved in the production of anti-microorganism substances, bacteriocins, toxins, organic acids, short chain fatty acid production, lowering of gut pH. These substances are responsible for inhibit the growth of other harmful microbes such as foodborne pathogens and spoilage organisms in GIT environment then lead to the death of the pathogen by creating antagonistic condition, and such action may result in the inactivation of toxins. Probiotic mode of effects are carried out based on microbial products which is determine a specific probiotic action and its effective application for the prevention or treatment of a certain disease by destruction of target cell. The third mechanism is the stimulation/modulation of specific and nonspecific immune response by T-cell activation, to cytokine production/throughout immune modulation by inducing phagocytosis and IgA secretion, modifying T-cell responses, enhancing Th1 responses, and attenuating Th2 responses. This mode of action is most likely important in the prevention and therapy of infectious diseases (Hebraud *et al*., 2011). Probiotic bacteria can exert an immune modulatory effect. These bacteria have the ability to interact with epithelial and dendritic cells (DCs) and with monocytes/macrophages and lymphocytes. In various strategies they are interact and modulate the immune system in a good manner (Bermudez-Brito *et al*., 2012). The immunological advantages of probiotics can be because of activation of local macrophages and modulation of IgA production locally and

systemically, to changes in pro/anti-inflammatory cytokine profiles, or to the modulation of response towards food antigens (Abatenh *et al*., 2018).

(i) It stimulates and modulates immune response.

(ii) It normalizes intestinal microflora by colonization resistance and controls irritable bowel syndrome and other inflammatory bowel diseases.

(iii) They have metabolic effects like-bile salt deconjugation and secretion, lactose hydrolysis, reduction in toxigenic and mutagenic reactions in gut, Supply of nutrients to colon epithelium (Shyamala *et al*., 2016).

2.5 Effect of Lactic Acid Fermentation on physico-chemical characteristics and Microbial quality of probiotic juices

2.5.1 pH

Yoon *et al*. (2004) have reported that lactic acid cultures (*Latobacillus acidophilus* LA39, *Lactobacillus plantarum* C3, *Lactobacillus casei* A4, and *Lactobacillus delbrueckii* D7) reduced the pH value to 4.1 or below of tomato juice after 72 h fermentation. Similarly, in another study, Yoon *et al*. (2005) reported that *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus delbrueckii*, *Lactobacillus plantarum* reduced the pH value of beet juice from 6.3 to 3.7, 4.1, 5.0 and 5.0 after 72 h of fermentation, respectively. Yoon *et al*. (2006) found that the pH values at the end of cabbage juice fermentation were 3.5, 3.6 and 3.6 mg/mL for *L. casei*, *L. delbrueckii*, and *L. plantarum*, respectively. Similarly, *L. plantarum* and *L. casei* reduced the pH value of roselle juice from 4.74 to 3.91 and 3.90 after 72 h fermentation at 30 °C, respectively (Vijaya Kumar *et al*., 2015). In another study, Guo *et al*. (2009) documented that during fermentation, *L. casei* was able to reduce the pH

value of milk to 5.59 after 24 h and after 28 days the pH further declined 4.60. Similarly, Hassan *et al*. (2012) reported that pH value of fermented rice and millet beverages decreased markedly to 4 at 8 h until 12 h and further decreased afterward. The time required to reach pH 4.5 for fermented plain rice beverage samples was recorded as 16 h while it was reduced to 10 h for fermented plain millet beverage.

2.5.2 Titratable acidity

Thakur, (2015) reported that lactic acid (LA) was the major end product of the fermented cabbage juice, attaining the concentrations of 9.69, 12.2 and 6.97 g/l LA for *L. casei*, *L. delbrueckii,* and *L. plantarum*, respectively. Further, probiotic bacteria could tolerate high acid medium and survived during fermentation process (Tayo & Akpeji, 2016). Yoon *et al*. (2004) found that lactic acid cultures (*Latobacillus acidophilus* LA39, *Lactobacillus plantarum* C3, *Lactobacillus casei* A4, and *Lactobacillus delbrueckii* D7) increased the acidity to 0.65% or higher of tomato juice after 72 h of fermentation. Similarly, Yoon *et al*. (2005) found that *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus delbrueckii* and *Lactobacillus plantarum* increased the titratable acidity in term of lactic acid of beet juice from 0.13 to 0.98, 0.56, 0.25 and 0.23 % after 72 h of fermentation respectively. *L. plantarum* and *L. casei* increased the acidity in term of lactic acid of roselle juice from 0.25 to 0.44 and 0.52% after 72 h fermentation at 30 °C, respectively as observed by Hebraud *et al*. (2011).

2.5.3 Sugar

It was observed that the lactic acid cultures rapidly fermented tomato juice and reduced the level of sugar (Yoon *et al*., 2004). *L. plantarum* consumed the sugar at a much faster rate than *L. acidophilus, L. casei*and *L. delbrueckii*. Such as *L. plantarum* and reduced the sugar

level from an initial value of 32.4 mg/mL to 25.2, 21.0, and 19.3 mg/mL after 24, 48, and 72 h fermentation, respectively. Yoon *et al*. (2006) reported that concentrations of sugar at the end of cabbage juice fermentation were 36.5, 19.33 and 28.35 mg/mL for *L. casei*, *L. delbrueckii*, and *L. plantarum*, respectively which were further decreased after fermentation from the initial value. Similarly, Hassan *et al*. (2012) found that *L. plantarum* and *L. casei* reduced the sugar level of roselle juice from 9.66 to 1.82 and 4.07mg/mL after 72 h fermentation at 30 °C respectively

2.5.4 Ascorbic acid

L-ascorbic acid is an indicator of the sparingness of the technological process, the concentration of this acid was also measured in the end products. It was concluded that about 20 to 70% of the initial content of L-ascorbic acid remains preserved in the end-products, depending on the used method of processing (Yoon *et al*., 2004). Kohajdova *et al*. (2006) reported that 43 % and 56 % of the original content of L-ascorbic acid was preserved at the end of fermentation of tomato juice and cabbage juice respectively.

2.5.5 Viable cell counts

Shah (2000) reported that the viability of probiotic organisms was dependent on the level of oxygen in products, oxygen permeation of the package, fermentation time, and storage temperature. Yoon *et al*. (2004) found that lactic acid cultures (*Latobacillus acidophilus* LA39, *Lactobacillus plantarum* C3, *Lactobacillus casei* A4, and *Lactobacillus delbrueckii* D7 increased the viable cell counts of tomato juice (CFU) from 1.0 to 9.0×10^{9}/ mL after 72 h fermentation. Similarly, Yoon *et al*. (2006) in another study reported that *L. casei*, *L. delbrueckii*, and *L. plantarum* grew well on cabbage juice and reached nearly $10x10^{8}$ CFU/mL after 48 h of fermentation at 30 °C. The cell number of *L. plantarum* in roselle

juice increased from $3.3x10^7$ to $4.6x10^8$ CFU/mL and cell number of *L. casei* from $8.5x10^7$ to $2.7x10^8$ reported by Hassan *et al*. (2012) in another study. Pereira *et al*. (2010) observed that cashew apple juice inoculated with 7.30 and 7.48 Log CFU/mL of *L. casei*, presented a fast growth. At 6 h of fermentation viable cell counts of 8.04±0.00 and 8.08±0.00 CFU/mL, respectively, was observed. Similarly, Pakbin *et al*. (2014) studied that probiotic lactic acid bacteria grew well in peach juice reached nearly 10×10^9 CFU/mL, after 48 h of fermentation at 30 ºC and was capable of consuming more sugar decreasing pH decrease and production of lactic acid during fermentation.

2.5.6 Antimicrobial activity

Lactic acid bacteria produced various compounds such as organic acids, diacetyl hydrogen peroxide, and bacteriocins or bactericidal proteins during lactic fermentations Costa *et al*. (2013) found that bacteriocins produced by lactic acid bacteria, such as nisin, inhibited not only closely related species but were also effective against food-borne pathogens and many other Gram-positive spoilage microorganisms. Similarly, Vieco-saiz *et al*., (2019) revealed that the LAB inhibited all the pathogenic bacteria and the inhibition was scored positive in the study when the width of the clear zone around the colonies of the producer strain was 0.5 mm or larger. Vantsawa *et al*. (2017) reported that inhibition of *Escherichia coli* and *Salmonella* under conditions that disrupt the outer membrane, including truncated lipopolysaccharides (LPS), low pH and high salt concentrations. Vijaya Kumar *et al*, (2015) studied the activity of LAB on some Gram positive and negative pathogenic bacteria such as *E.coli, Pseudomonas aeroginosa, Klebsiella pneumonia, Staphylococcus aureus* and *Bacillus cereus* and the inhibition zones were in the range of 1.4 to 2.8cm Similarly, cell free supernatant of the Lactic acid bacteria inhibited the growth of all organisms (*E.*

coli, *Klebsiella*, *Pseudomonas*, *Streptococcus*, *Proteus*) (Saranya and Hemashenpagam, 2011). The antimicrobial effect of lactic acid was found to be due to un dissociated form of acid that penetrated the membrane and liberated hydrogen ion in the neutral cytoplasm thus, leading to inhibition of vital cell functions.

2.6 Health Benefits of Probiotics

The major beneficial effects are correlated against various disease conditions; they have a colossal criticalness and application in controlling different kinds of microbial infections (Abatenh *et al*., 2018).

2.6.1 Probiotics and allergy

Allergies are misguided reactions of the immune system in response to (what should be harmless) particles. Probiotics treat allergies by healing your damaged digestive system, which decreases inflammation, stabilizes your immune system, and strengthens your gut lining. An allergy is a hypersensitivity reaction initiated by immunological mechanisms. Probiotics modify the structure of antigens, reduce their immunogenicity, intestinal permeability and the generation of pro-inflammatory cytokines that are eminent in patients with a diversity of allergic disorders (Shyamala *et al*., 2016). *Lactobacillus* GG and *L. rhamnosus* GG is alleviating the symptoms of food allergies at the same time have significant role in reduction of risk for developing allergic disease (Licciardi *et al*., 2013). Already known strategies to solve allergic disorder by prevention of antigen translocation into blood stream, improve mucosal barrier function and prevent excessive immunologic responses to increased amount of antigen stimulation of the gut (Abatenh *et al*., 2018)

2.6.2 Probiotics and blood pressure

It has also been demonstrated that probiotics and their products can improve Blood pressure through mechanisms including improving total cholesterol and low-density lipoprotein cholesterol levels (Patel *et al*., 2010). Reducing blood glucose level and insulin resistance, regulating the renin–angiotensin system and significant reduction takes place in blood or serum cholesterol when cholesterol is elevated (Guo *et al*., 2011). Interestingly, probiotic supplementation might positively help in reducing Blood pressure in the hypertensive conditions. *Lactobacillus helveticus, Saccharomyces cerevisiae*, *Lactobacillus rhamnosus* GG, *Lactobacillus casei*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Lactobacillus bulgaricus, Bifidobacterium breve, Bifidobacterium longum Streptococcus thermophiles, Lactobacillus delbrueckii* ssp. *Bulgaricus, Lactobacilluskefiri* are the common one used for anti-hypertension (Rerksuppaphol and Rerksuppaphol, 2015; Ekhlasi *et al*., 2017).

2.6.3 Probiotics and inflammatory bowel disease

Incorporation of probiotic bacteria has an ability to become stable the immunological barrier in the gut mucosa by declining the generation of local pro-inflammatory cytokines. Probiotics is used for treatment of the inflammatory bowel disease, such as ulcerative colitis, Crohn's disease and Pouchitis. Potential mechanisms include suppression of growth or epithelial binding and invasion by pathogenic bacteria, production of antimicrobial substances, improved epithelial barrier function, and immune-regulation. The effects of probiotic are probably both strain-dependent and dose dependent (Hassan *et al.,* 2012).

2.6.4 Probiotics and urogenital infections (Bacterial *vaginitis*)

Bacterial *vaginosis* is an abnormal vaginal condition that is characterized by vaginal discharges and results from an overgrowth of typical bacteria in the vagina. A urinary tract infection is an infection involving the kidneys, ureters, bladder, or urethra. These are the structures that urine passes through before being eliminated from the body. Urogenital infection occurs due to change in vaginal environmental in which *Lactobacilli* decrease in concentrations or absent. *Lactobacillus* spp, are the prominent microbial factors that governs the presence, growth, colonization and persistence of non- endogenous microorganisms in vagina (Ya *et al*., 2010). As the *Lactobacillus* spp. count decreases, the protection provided by them against pathogens also decreases. It is also proposed that *lactobacilli* produce biofilms, which cover the urogenital cells. *Lactobacilli* use in bacterial vaginosis is supported by positive results obtained in clinical trials (Okereke *et al*., 2016). Probiotic capsules for example *Lactobacillus rhamnosus, Lactobacillus crispatus, Lactobacillus gasseri, Lactobacillus vaginalis, Lactobacillus acidophilus, Lactobacillus reuteri and Streptococcus thermophilus* are effectiveness for recurrent bacterial vaginosis prevention (Worku *et al*., 2019).

2.6.5 Probiotics and liver diseases

Microflora resident in intestinal lumen plays a significant role in hepatocytes function. Alterations to the type and amount of microorganisms that live in the intestinal tract can result in serious and harmful liver dysfunctions such as cirrhosis, nonalcoholic fatty liver disease, alcoholic liver disease, and hepatic encephalopathy (Lunia *et al*., 2014). Probiotic is used as a novel treatment strategy against liver disease in a mechanism of regulation,

restoration and alteration of gut micro flora and immune function (Cesaro *et al*., 2011). Probiotics are useful in the treatment of chronic liver diseases as they block entry of microorganisms to blood flow and ultimately to liver by increasing the strength of intestinal barrier (Cesaro *et al*., 2011).

2.6.6 Probiotics and cholesterol assimilation

Probiotic strains, particularly lactic acid microscopic organisms (bacteria) have a noteworthy part to play in the cholesterol by bringing down the mechanism (Abatenh *et al*., 2018). The cholesterol levels can be cut down direct or indirect by using probiotics. Direct mechanism involves inhibition of denovo synthesis or decrease in the intestinal absorption of dietary cholesterol. The decrease in dietary cholesterol retention can be diminished by three ways -assimilation, binding or by degradation. Probiotic strains absorb the cholesterol for their own particular digestion. Probiotic strains can attach to the cholesterol particle, and they are capable for debasing cholesterol to its catabolic products (Vaishnavi *et al*., 2016). The cholesterol level can be decreased in an indirect way by deconjugating the cholesterol to bile acids (Bordoni *et al*., 2013.) Hypercholesterolemia (elevated blood cholesterol level) is considered a major risk factor for the development of coronary heart disease. Therefore, lowering the serum cholesterol level is important to prevent the disease. The cholesterol removing ability of LAB isolates was assessed in vitro and in vivo mechanisms. *Lactobacillus pentosus* LP05, L. *brevi*s LB32, L. *reuteri* and L. *plantarum* are powerful (Abatenh *et al*., 2018).

2.7 Why we Need Non-dairy Probiotics

Previously, health benefits of probiotics were fulfilled by milk and other dairy products; however lactose intolerance, cholesterol content and allergic milk proteins are limiting factors in growth of dairy probiotics (Panghal *et al.*, 2018). Total 75 % of the world's population is suffering from lactose intolerance (Silanikove *et al.*, 2015). Lactose intolerance is basically absence of lactase enzyme production by intestinal brush which hydrolyse lactose into absorbable sugars (glucose and galactose) to provide energy. Lactose is crucial first carbohydrate for promoting the health of new born (Wahlqvist, 2015). During the postnatal period, the activity of the intestinal enzyme (lactase) is maximal amongst most infants which further diminish with the age. Nevertheless, among two to twelve year old children, the segregation occurs into two distinct groups, namely "lactase non-persistence group" having hypolactasia and a "lactase-persistence group" who pertain their neonatal level of lactase activity even after the phase of infancy. The commonly observed symptoms in lactose intolerant people are like bloating gastric pain and cramps, gas production in gastrointestinal track and diarrhoea. Probiotics helps in release of β-galactosidase in small intestine which assists in lactose digestion/breakdown. So, probiotics intake can lessen the severity of lactose intolerance, but the effectiveness depends on number of cells in the product and amount of lactose present. Besides this with an increase in vegetarian consumers in both developed and developing countries, there is also a high demand for plant based probiotic products. High cholesterol content dairy products, significant number of lactose intolerant people and economics reasons for the developing countries also necessitates the search for dairy alternatives with good nutrients along with health promoting factors for example fruits, vegetables, cereal, and legume (Panghal *et al.*,

2017b) and from products which are lack of cholesterol content however rich in protein, starches, minerals, fiber, vitamins and disease preventing antioxidant contents. Non-dairy probiotic foods are used as therapeutic treatment product for the people having lactose intolerance (Deng *et al.*, 2015).

2.8 Fruit Based Probiotic Food Products

Fruits are an ideal medium for the functional foods and have more nutritional values due to the essence of various phyto-chemicals, antioxidant contents, no cholesterol, vitamins, mineral content and dietary fibres (Montet *et al.*, 2014). Fruits are healthy, refreshing and have good taste and flavour profile and can be suitable for probiotics (Panghal *et al*., 2017 b). Highly perishable and short shelf life of fruits necessitates for immediate processing to reduce the post-harvest losses and probiotic product development can be an approach to enhance availability and market value of product (Panghal *et al*., 2017 a). Dairy allergens are also preventing some people to consume dairy probiotics and fruit and vegetables are free from dairy allergens, lactose and cholesterol and so are suitable substrate (Luckow and Delahunty, 2004 a). Fruit based probiotic products are made by pineapple, cranberry, strawberry, sweet lime, mango, grapes, cashew apple, olive, and oranges (Panghal *et al*., 2018)

2.8.1 Grapes probiotic

Hardaliye is a grape fruit based non-alcoholic, non-dairy probiotic, traditional beverage of Turkey, fermented with mostly lactic acid bacteria along with addition of crushed mustard seeds and benzoic acids. Eteric oil from mustard seed affects yeast and also provides a characteristic flavour to the finished product. Benzoic acid inhibits or decrease alcohol

production by affecting the yeast, the pH of hardaliye varies from 3.21 to 3.97 (Malganji *et al.*, 2015) which can be increased slightly with the addition of clove or ginger. The color intensities, phyto-chemical and antioxidant contents of hardaliye changes depending on grape varieties and preparation process (Coskun, 2017). The probiotic bacteria used in fermentation of hardaliye are *L. casei* subsp. *pseudoplantarum* and *L. paracasei* subsp. *paracasei* predominantly followed by *L. sanfranciscensis* (formerly known as *L. sanfrancisco*), *L. brevis, L. pontis, L. acetotolerans*, and *L. vaccinostercus*. Guven and Aksoy (2009) studied fermentation of grape juice by lactic acid bacteria (*L. plantarum, L. delbrueckii,* and *L. rahamnosus*). They found that lactic acid production was up to 0.27% which reduced the pH up to 3.7 (Thakur and Joshi, 2017).

2.8.2 Peach probiotics

Peach juice can be well fermented by *L. delbrueckii* and are appropriate to produce a probiotic beverage (Pakbin *et al*., 2014). The fermented probiotic peach juice can serve a healthy beverage for lactose-allergic consumers. Chen *et al*. (2012) made Yan-taozih, peach pickle by lactic acid bacteria like *L. brevis, L. lactis, Weissellacibaria, W. paramesenteroides, W. minor, Leuconostoc mesenteroides,* and *Enterococcus faecalis*. Pickle can be stored for a longer time at 6-10 ^{o}C temperature.

2.8.3 Mango probiotic juice

Probiotic mango juice was done by lactic acid and other probiotic bacteria (Reddy *et al.*, 2015). Mango juice was fermented at 30 ^{o}C for 72 h and pH was reduced to 3.2 during fermentation. Probiotic lactic acid bacteria used were *L. acidophilus, L. delbrueckii, L. plantarum and L. casei* and among them *L. plantarum* utilized the sugar at fastest rate.

2.9 Cereals base probiotics food products

Cereals are also a substrate that has been used for the production of probiotic products since they contain substances that act as prebiotics and protect probiotic cells from the adverse conditions of the gastrointestinal tract (Kandylis *et al*., 2016). Consumption of cereal has been associated with the risk reduction of several chronic diseases. Presently, several researchers try to produce probiotic beverages based on single or multi cereals. Salmero´n *et al*., 2015 produced several beverages using starch-free extracts of flour–water suspensions prepared from oats, barley and malt with human derived *L. acidophilus*, *L. plantarum*, and *L. reuteri*. The produced beverages had pH values from 3.3 to 3.7 and high cells viability (10^8 CFU/mL). The beverage produced with malt and *L. acidophilus* exhibited higher acceptance from the consumers. In addition the results showed that the products with higher acetaldehyde concentrations and mid pH values had better acceptance during sensory evaluation (Kandylis *et al*., 2016).

2.9.1 Oats probiotic beverage

Oats have also been used for the production of probiotic beverage. This product had similar characteristics with the traditional yogurt-like beverages, while the fermentation with *L. plantarum* increased the polyphenol content and the antioxidant activity Luana *et al*., 2014 in a similar study a new probiotic product was produced after fermentation of oat milk with *L. reuteri* and *S. thermophilus* (Bernat *et al.,* 2014). The final product was capable to retain starters' survival above 10^7 CFU/ mL even after storage at 4 ºC for 28 days (Kandylis *et al.*, 2016).

2.10 Principles of Probiotic Systems

Besides surviving the storage conditions, the strains used in probiotic foods also have to survive in the gastrointestinal tract and reach the intestines alive. Also, the microorganisms' security and functionality must be evaluated in the selection process for their application in humans. Among the safety requirements, the probiotics should not be pathogenic, do not display toxicity, and their origin must be the gastrointestinal tract of healthy humans. The ability to adhere to the intestinal mucosa, antagonist activity, antimicrobial substances production, and resistance to gastric juice and bile salts are some of the characteristics evaluated during microorganism selection. To exert their health benefits, the minimum concentration of live probiotic bacteria at the end of the product shelf life should be around 10^7 CFU/mL (Ding and Shah, 2008). To make sure the probiotic food will accomplish the desired effect, The Food Agriculture Organization of United Nations (FAO) and The World Health Organization (WHO) established guidelines for the evaluation of probiotics in foods (Fernandes Pereira and Rodrigues, 2018).

2.11 Storage of Probiotic Juices

Storage study of probiotic juice has been done by many researchers to study the effect of cold storage on viable cell counts of lactic acid bacteria of fermented fruit juices.

Yoon *et al*. (2005) found that the viable cell counts of *Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus delbrueckii, Lactobacillus plantarum* in the fermented beet juice still remained at 10^6-10^8 CFU/mL except *L. acidophilus* after 4 weeks of cold storage at 4 ºC. Yoon *et al*. (2006) also studied that after 4 weeks of cold storage of fermented cabbage juice at 4 °C, the viable cell counts of *L. plantarum* and L. *delbrueckii*

were still 4.1 x 10^7 and 4.5 x 10^5 mL -1, respectively. *L. casei*, however, did not survive the low pH and high acidic conditions in fermented cabbage juice and lost cell viability completely after 2 weeks of cold storage at 4 ºC. The cell number of fermented roselle juice was also reduced to approximately10^4 CFU/mL after 3 weeks of cold storage at 7 °C (Shyamala *et al*., 2016). Kohajdova *et al*. (2006) have reported that colour, turbidity, sediment and overall appearance are the most important parameters to evaluate the appearance of lactic acid fermented fruit and vegetable juices. Sensory characteristics of probiotic blackcurrant juice were described as perfumery, dairy in odour and sour and savoury in flavour (Luckow and Delahunty, 2004). Kohajdova *et al*. (2006) have also reported that sensory characteristics of probiotic celery juice were pleasant, sweet-sour taste, without serve any acidity.

2.12 Commercially Available Probiotic-based Fruit Juices

In 2012, the Heinz's Golden Circle Healthy Life launched two flavors of probiotic fruit juices in Australian market, Apple Mango Juice with Probiotic Cultures, a blend of cloudy apple juice, tropical mango juice and Breakfast juice. The probiotic microorganism is *L. paracasei* 8700:2 and *L. plantarum* HEAL 9 (Fernandes Pereira and Rodrigues, 2018).

GoodBelly is a brand of Next Foods USA. GoodBelly has seven different flavors of fruit juice containing *L. plantarum* 299v (LP299V): Mango Blueberry Acai Pomegranate Blackberry Tropical Green, a blend of fruits and greens such as spinach, alfalfa grass, Tropical Orange a blend of orange juice and tropical fruit juices and Coconut Water. Naked Juice is a brand of Pepsi Company (PepsiCo). Their probiotic product is called Probiotic Machine Tropical Mango, and it is a blend of apple juice, mango puree, orange juice,

pineapple juice and banana puree, The probiotic strain used is *Bifidobacterium* (Fernandes Pereira and Rodrigues, 2018). ProViva is the Danone Nordic brand of probiotic fruit juices, ProViva's strain is *L. plantarum*, the same used in GoodBelly. ProViva is a complete line of probiotic fruit-based beverages. The regular product is available in the following flavors: Passion Orangea fruit mix of juice and pulp of orange, grape juice, passion fruit juice, and banana puree.

Another product is ProViva Super Fruit, which are shots with five-times as much living bacterial culture than ProViva regular fruit drinks, available in two flavors: Blackcurrant and Blueberry Pomegranate Yumberry. ProViva Active is an energy drink with additional proteins, carbohydrates, vitamins, and minerals, available in the following flavors: Blueberry Raspberry Lemon and Tropical. The last product of Danone Nordic probiotic fruit-based beverages is ProViva Lemonade, available in two flavors: Raspberry Lemon and Lemon Lime (Fernandes Pereira and Rodrigues, 2018).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Description of the Study Area

The sample of cow milk was collected from Bosso Local Government Area, Minna Niger State, Nigeria. The map of Bosso, Minna showing the sample area is shown in Figure 3.1. It was collected under good condition from healthy animal to avoid contamination, which can influence the lactic flora. The sample was collected in sterile bottle and transported quickly to the laboratory for analysis. The analysis was done at the of microbiology laboratory of Federal University of Technology Minna, Niger State. Sterilization, serial dilution and preparation of media were done according to Harrigan and MacCance (1976).

3.2 Growth Media of Bacterial strains (Lactic Acid Bacteria)

deManRogosa Sharpe (MRS) agar and broth are used for the isolation, they are usually designated for anaerobes, 67grams the Suspended powder was dissolved in 1 Liter of distilled water and autoclaved at 121 ºC for 15minutes for the isolation of the bacteria.

3.3 Isolation and Characterization of Lactic Acid Bacteria

Lactic acid producing bacteria were isolated from fresh cow milk, Serial ten-fold dilutions for the cow milk samples were prepared.0.1mL aliquot of 10^{-5} dilution was pour plated on MRS agar, Isolation of organisms was done with the pour plate method using molten MRS agar. After solidification, they were incubated anaerobically in an anaerobic jar (containing a gaspak used to evacuate all traces of oxygen thereby creating an environment having only carbon IV oxide) at 30 ºC for 48 h, the purity of the isolated organisms was checked by streaking again on MRS agar, and pure cultures were selected and stored on slant.

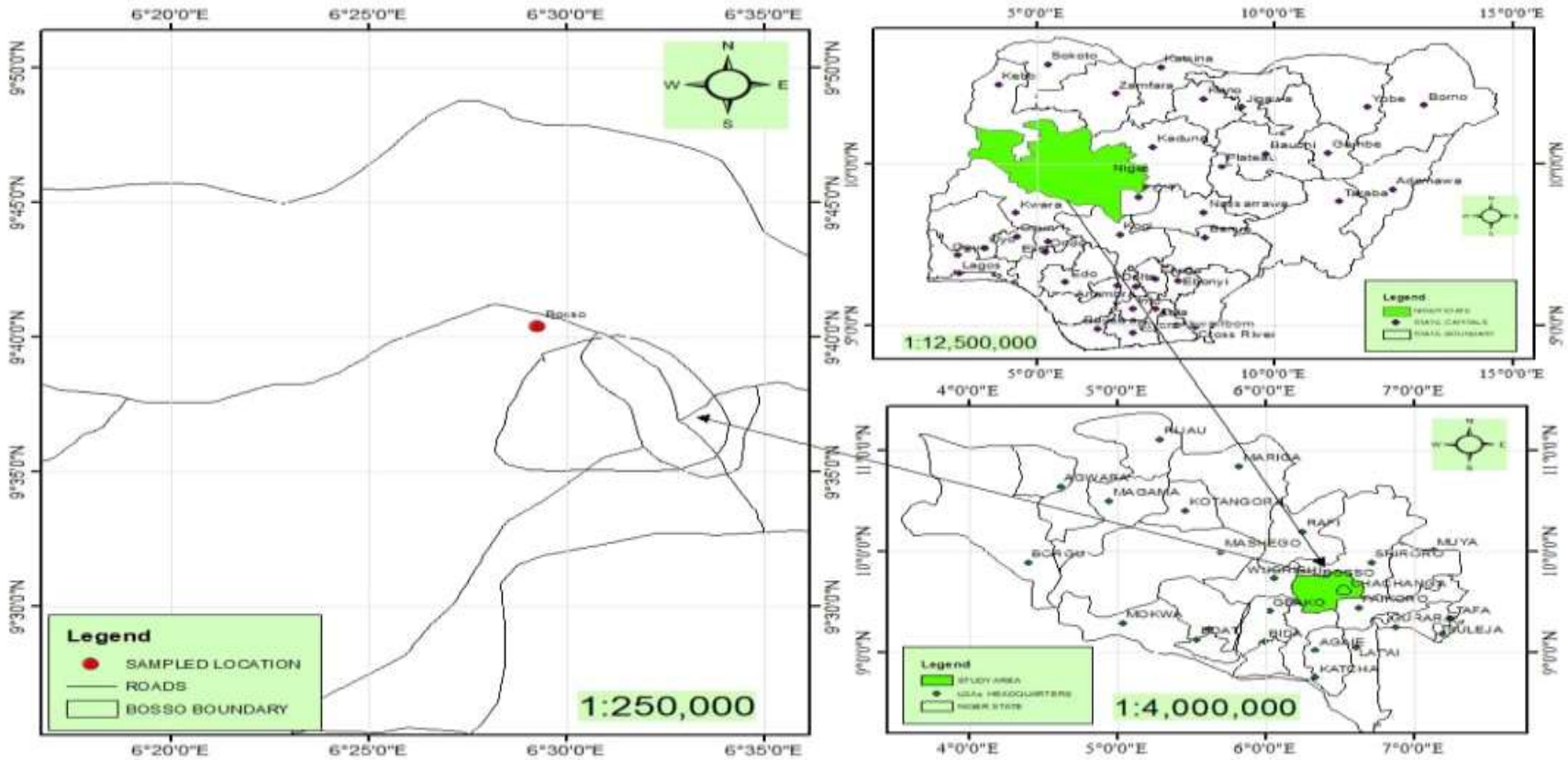

Figure 3.1: Map of Bosso, Minna Niger State Nigeria, showing the collection of sample area (Fresh cow milk)

Source: Remote Sensing/ Geographical information system (GIS) laboratory, Geography department, FUTMINNA (2019).

3.4 Identification of Lactic Acid Bacteria

The pure strain was streak cultured on MRS medium for 48 h at 30 ºC, the colony size and shape were observed and tested microscopically by Gram staining, catalase, motility test, oxidase test, slime production test, nitrate reduction test, indole test, methyl red test, 6.5 % NaCl growth test, pH gradient test and sugar alcohol fermentation test were used for the identification of the Lactic Acid Bacteria.

3.4.1 Gram's staining test:

Gram's stain was done using the method described by Harrigan and McCance (1976). A heat-fixed smear was prepared from a 24 h culture in the usual way from MRS broth and stained with crystal violet solution for 2 minutes. Then, rinsed rapidly with water and Gram's iodine solution was added and left for 1 minute. The iodine was poured off and blotted dry. The slide was washed with 95 % ethanol for 15 seconds until no more violet stain runs from the slide.

The slide was rinsed under the tap and stained with dilute carbolfuschin solution for 20 seconds. The slide was washed well and blotted dry. The slide was viewed under a microscope using oil immersion.

3.4.2 Motility test:

The motility of isolate was determined using the method described by Harrigan and McCance (1966).A small drop of suspension was placed on a slide and covered with a cover slip (glass). The preparation was not made too thick. The preparation was examined

microscopically for motile organisms using the 10 x and 40 x objectives. The movement of small motile bacteria must be distinguished from the on-the-spot vibratory movement (Brownian movement) which is shown by all micro-organisms and particles when suspended in a fluid.

3.4.3 Slime production test:

For slime production, strains were inoculated on the suitable media (MRS broth) and incubated at 42 ºC for 24 h. Ropiness of colonies was tested on agar surfaces it was tested with a loop to observe the formation of slime (Zambou *et al*., 2007).

3.4.4 Catalase test:

Catalase test was done using the method described by Harrigan and McCance (1976). It was done by dripping two drops of hydrogen peroxide (H_2O_2) 3 % of 24 h aged cultures on a clean glass slide. Catalase test positive reaction characterized by the formation of oxygen bubbles that indicate the bacteria produce the catalase enzyme which converts H_2O_2 to water (H_2O) and oxygen (O_2) (Vantsawa *et al*., 2017).

3.4.5 Indole test:

Freshly prepared Kovac's indole reagent was used. Two grammes of 4-dimethylaminobenz-aldehyde were dissolved in 30 mL of isoamyl alcohol (3-methyl-1-butanol) solution. Then 10 mL of concentrated hydrochloric acid was added and mixed well according to Harrigan and McCance (1976). A loopful of the pure isolates was grown in sterile peptone water for 48 h at 37 ºC. The two days old cultures were used by the addition of 0.2 mL freshly prepared Kovac's reagent shaken and allowed to stand for 5 minutes. The

appearances of a red coloration on the amyl alcohol layers indicate positive indole test and negative result remains as a yellow layer on the interface (Teneva-Angelova and Beshkova, 2016).

3.4.6 Test for sugar fermentation (Triple Sugar Iron Agar)

About 65g of the dehydrated Triple Sugar Iron medium was dissolved in 1 liter of distilled water and homogenized by boiling. The medium was mixed well and dispersed into cotton-plugged test tubes. These tubes were sterilized by autoclaving at 151 pressure and 121 ºC for 15 minutes. The medium was allowed to set in sloped form with a butt of about 1inch long. The slants were inoculated with test isolate by streaking the slope and stabbing the butt. The test tubes were incubated at 37 ºC for 24 h. The results were taken immediately after incubation as follows: Yellow slant with yellow butt →Fermentation of lactose and glucose by bacteria

Red slant and yellow butt → Fermentation of glucose and not lactose and sucrose(Science, 2018).

3.4.7 Oxidase test

One percent of oxidase reagent was prepared by the dissolution of 0.1 g of tetramethyl phenylenediamine hydrochloride in 10 mL of distilled water. A drop of the freshly prepared oxidase reagent was added to a 24 h old culture agar plates. A positive test was observed by the production of a purple colouration within 5-10 seconds while a delayed purple colour (10-16 seconds) indicated negative test (State *et al.*, 2011).

3.5 Molecular Identification of the Isolates

3.5.1 DNA extraction

DNA was extracted using the protocol stated by (Saitou and Nei, 1987). Briefly, Single colonies grown on medium were transferred to 1.5 mL of liquid medium and cultures were grown on a shaker for 48 h at 28 ºC. After this period, cultures were centrifuged at 4600 g for 5 minutes; the resulting pellets were re-suspended in 520 µL of TE buffer (10 mMTris-HCl, 1 mM EDTA, pH 8.0). Fifteen microliters of 20 % SDS and 3 µL of Proteinase K (20 mg/mL) were then added. The mixture was incubated for 1 hour at 37 ºC then 100 µL of 5 M NaCl and 80 µL of a 10 % CTAB solution in 0.7 M NaCl were added and votexed. The suspension was incubated for 10 minutes at 65 ºC and kept on ice for 15 minutes. An equal volume of chloroform: isoamyl alcohol (24:1) was added, followed by incubation on ice for 5 minutes and centrifugation at 7200 g for 20 minutes. The aqueous phase was then transferred to a new tube and isopropanol (1:0.6) was added and DNA precipitated at –20 ºC for 16 h. DNA was collected by centrifugation at 13000 g for 10 minutes, washed with 500 µL of 70 % ethanol, air-dried at room temperature for approximately three hours and finally dissolved in 50 µL of TE buffer.

3.5.2 Polymerase chain reaction

PCR sequencing preparation cocktail consisted of 10 µl of 5 x Go Taqcolourless reaction, 3 µl of 25 mM $MgCl_2$, 1 µL of 10 mM of dNTPs mix, 1 µL of 10 pmol each 27F 5'- AGA GTT TGA TCM TGG CTC AG-3' and - 1525R, 5′-AAGGAGGTGATCCAGCC-3′ primers and 0.3 units of Taq DNA polymerase (Promega, USA) made up to 42 µL with sterile distilled water 8 µL DNA template. PCR was carried out in a GeneAmp 9700 PCR

System Thermalcycler (Applied Biosystem Inc., USA) with a PCR profile consisting of an initial denaturation at 94 ºC for 5 minutes, followed by a 30 cycles consisting of 94 ºC for 30 seconds, 50 ºC for 60 seconds and 72 ºC for 1 minute 30 seconds and a final termination at 72 ºC for 10 minutes and chill at 4 ºC.GEL (Tamura *et al*., 2004)

3.5.3 Integrity test

The integrity of the amplified about 1.5 Mb gene fragment was checked on a 1 % Agarose gel ran to confirm amplification. The buffer (1XTAE buffer) was prepared and subsequently used to prepare 1.5 % agarose gel. The suspension was boiled in a microwave for 5 minutes. The molten agarose was allowed to cool to 60 ºC and stained with 3 µl of 0.5 g/mL ethidium bromide (which absorbs invisible UV light and transmits the energy as visible orange light). A comb was inserted into the slots of the casting tray and the molten agarose was poured into the tray. The gel was allowed to solidify for 20 minutes to form the wells. The 1XTAE buffer was poured into the gel tank to barely submerge the gel. Two microliter (2 l) of 10X blue gel loading dye (which gives colour and density to the samples to make it easy to load into the wells and monitor the progress of the gel) was added to 4 µL of each PCR product and loaded into the wells after the 100bp DNA ladder was loaded into well 1. The gel was electrophoresed at 120 V for 45 minutes visualized by ultraviolet trans-illumination and photographed. The sizes of the PCR products were estimated by comparison with the mobility of a 100 bp molecular weight ladder that was ran alongside experimental samples in the gel.

3.5.4 Purification of amplified products

The amplified fragments were ethanol purified in order to remove the PCR reagents. Briefly, 7.6 µL of Na acetate 3 M and 240 µL of 95 % ethanol were added to each about 40 µL PCR amplified product in a new sterile 1.5 µL tube eppendorf, mix thoroughly by vortexing and keep at -20 ºC for at least 30 minutes. Centrifugation for 10 minutes at 13000 g and 4 ºC followed by removal of supernatant (invert tube on trash once) after which the pellet were washed by adding 150 µL of 70 % ethanol and mix then centrifuge for 15 minutes at 7500 g and 4 ºC. Again remove all supernatant (invert tube on trash) and invert tube on paper tissue and let it dry in the fume hood at room temperature for 10-15 minutes then re-suspend with 20 µL of sterile distilled water and kept in -20 ºC prior to sequencing. The purified fragment was checked on a 1.5 % Agarose gel ran on a voltage of 110 V for about 1 hour as previous to confirm the presence of the purified product and quantified using a nano drop of model 2000 from thermo scientific.

3.8.5 Sequence analysis

The amplified fragments were sequenced using a Genetic Analyzer 3130xl sequencer from Applied Biosystems using manufacturers' manual while the sequencing kit used was that of Big Dye terminator v3.1 cycle sequencing kit. Bio- Edit software and MEGA 6 were used for all genetic analysis.

3.6 Survival under Conditions Simulating the Human GI Tract

In order to act as a probiotic in the gastrointestinal tract and to exert their beneficial effect on the host, the bacteria must be able to survive the acidic conditions in the stomach and resist bile acids at the beginning of the small intestine. Approximately 2.5 liter of gastric

juice and 1 liter of bile are secreted into the human digestive tract every day. Thus, it is essential for the bacteria to have protection systems to withstand the low pH in the stomach, digestive enzymes and bile in the small intestine(Argyri *et al*., 2013).

3.6.1 Resistance to low pH

The methods used and described are according to Maragkoudakis *et al*. (2006) and Zoumpopoulou *et al*. (2008). Bacterial cells from overnight (18 h) cultures were harvested, washed twice with PBS buffer (pH 7.2), before being re-suspended in PBS solution, adjusted to pH 2.5. Resistance was assessed in triplicates in terms of viable colony counts and enumerated on MRS after incubation at 37 ºC for 0, 0.5, 1, 2, and 3 h, reflecting the time spent by food in the stomach (Argyri *et al*., 2013).

3.6.2 Resistance to bile salts

Bacterial cells from overnight (18 h) cultures were harvested before being re-suspended in PBS solution (pH 8.0), containing 0.5 % washed twice with PBS buffer (pH 7.2), bile salts, Resistance was assessed in triplicates in terms of viable colony counts and enumerated after incubation at 37 ºC for 0, 1, 2, and 4 h reflecting the time spent by food in the small intestine (Argyri *et al*., 2013).

3.6.3 Bile salts hydrolysis

Fresh bacterial cultures were streaked in triplicates on MRS agar containing 0.5 % (w/v) taurodeoxycholic, the hydrolysis effect was indicated by different colony morphology (partial hydrolysis) from the control MRS plates, after 48 h of anaerobic incubation at 37 ºC (Hassanzadazar *et al.*, 2012).

3.7 Determination of the Survivability of the LAB Strain inside the Orange and Watermelon Probiotic Juice

The survivability of the LAB strain in the probioticated juice samples was determined using a pour plate technique. The stored samples were pour plated at weekly intervals. Sample (1 mL) was inoculated on MRS agar plates and the plates were incubated at 37 ºC for 48 h. Viable cell counts were recorded by counting the visible colonies on the culture medium and the number was multiplied by the reciprocal of the dilution factor and expressed as colony forming units (CFU) (Tayo and Akpeji, 2016).

3.8 **Antagonistic Activity of Probioticated Substrate**

The antagonistic activity of the probioticated substrate against pathogenic bacteria *Escherichia coli* was investigated using agar well diffusion. The test pathogens containing 2.8×10^7 CFU/mL was seeded on a sterile molten nutrient agar. After solidification, wells were bore on seeded agar plates, and the probioticated juice sample was introduced into the wells. The plates were first incubated at 4 ºC for 60 minutes to allow the test material to diffuse in the agar and were then incubated at 37 ºC for 18 h. After incubation, the diameter of the clear zone was measured in centimeters from the centre of the well (Tayo and Akpeji, 2016).

3.9 Production of Probiotic Fruit Juices

To conduct the lactic acid fermentation, 5 % inoculum of both bacteria (*Lactobacillus plantarum* and *Lactobacillus paracasei* at the concentrations of 10^6 CFU/mL were added into pasteurized watermelon and orange juice under sterile condition. Fermentation was conducted for 72 h at 30 ºC. After completion of fermentation, the best treatment from each

fruit was selected for cold storage studies. Complete pictorial representation of preparation of the watermelon and orange probiotic juices are shown in Plate I and II respectively.

3.9.1 Extraction of fruits juices and their physico-chemical evaluation

The fruit juices were extracted by following the standard method (Tsegay, 2020).

3.9.2 Pasteurization of juices

The fruit juices were pasteurized at 70 ºC temperature for 10 minutes immediately followed by hot filling bottling and pasteurization of filled bottles.

3.10 Viable Cell Count (CFU/mL)

Viable cells (CFU/mL) were determined by the standard plate count method using MRS medium. After a series of appropriate dilutions, the samples were plated on MRS agar plates and incubated at 37 ºC for 48 h. The grown colonies were manually counted, and this number was multiplied by inverse of the plate dilution, resulting in bacterial count in colony forming units per mL of the watermelon and orange juices (CFU/mL). After completion of fermentation, the fermented samples were stored at 4 ºC for 4 weeks and the viability of probiotic bacteria was measured during storage time. The microbial population was measured at weekly intervals and expressed as CFU/mL. All of these determinations were carried out in three replications (triplicate).

3.11 Fermentation of Orange and Watermelon Juices:

To conduct the lactic acid fermentation, 5% inoculum of both bacteria (*Lactobacillus plantarum* and *Lactobacillus paracasei*) and their mixed (2.5 % each) culture at the concentrations of 10^6 CFU/mL were added in the pasteurized orange and watermelon juices

under sterile condition (Thakur, 2015). After inoculation of the bacteria culture, fermentation of fruit juices was carried out for 72 h at 37 ºC temperature in an incubator. On the basis of the physico-chemical and microbiological changes, the standization of probiotic orange and watermelon juices was carried out. The best treatment was selected for further cold storage studies.

Watermelon fruits

Cut in halves

Separate the segments of Fruits

Extraction of the juice with blender

Juice

Pasteurized Juice (10 mins) at 70 ºC

Addition of 5 % *Lactobacillus plantarum* and *Lactobacillus paracasei*

Fermentation (72 h) at 37 ºC

Kept the fermented juice in storage (4 ºC)

Plate I. Production process involved in probiotic watermelon juice

Orange fruits

Cut in halves

Separate the segments of Fruits

Extraction of the juice with blender

Juice

Pasteurized Juice (10 mins) at 70 ºC

Addition of 5 % *Lactobacillus plantarum* and *Lactobacillus paracasei*

Fermentation (72 h) at 37 ºC

Kept the fermented juice in storage (4 ºC)

Plate II. Production process involved in probiotic orange juice

3.12 Sensory Evaluation

Ten panelists were used in this study to assess the sensory properties of the produced probiotic juice samples through descriptive evaluation. The panelists were asked to rate the different juice samples using the Hedonic rating scale. 5 point hedonic rating test was performed to assess the degree of acceptability of these juices as follows: (5= like a lot, 4= like a little, 3= neither like nor dislike 2= dislike a little and 1= dislike a lot.

3.13 Data Analysis

All experiments were carried out in triplicate and each sample was analyzed in duplicate, all results of this study are reported as mean of three replicates, one way analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) was used to provide the significant levels for the difference between the pair of means for all physico-chemical analysis, they were determined by using SPSS (Statistical Package for the Social Science) software. The least significant difference was calculated at 95 % level of significance ($P < 0.05$); differences at $p < 0.05$ were considered as significant. All experiments were carried out in triplicate and each sample was analyzed in duplicate. The results were expressed as mean ± S.E (standard Error of mean), figures and tables were used to represent data.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Results

4.1.1 Morphological characteristics of Lactic Acid Bacterial isolates

The morphological test results of the Lactic Acid Bacteria is showed in Tables 4.1 Therefore, preliminary identification confirmed that the strain might be *Lactobacillu*s.

Table 4.1 Morphological Characteristics of Lactic Acid Bacterial Isolates

Characteristics	**LAB 1**	**LAB 2**
Colony Morphology	Creamy-white, circular, smooth, low convex	White to very light yellow, smooth, lens or diamond shape
Cell shape	Rods with sub-terminal ellipsoidal spores	Rod
Mobility	Non motile	Non motile

Keys: LAB1- Lactic Acid Bacteria 1, LAB 2 – Lactic Acid Bacteria 2.

4.1.1.1 *Biochemical test results of Lactic Acid Bacterial Isolates*

The biochemical test results of Lactic Acid Bacteria is showed in Tables 4.2, the strains were suspected to be *Lactobacillus plantarum* and *Lactobacillus paracasei.*

TABLE 4.2 Biochemical characteristics of Lactic Acid Bacteria isolates

TEST	Gram staining	Catalase test	Oxidase test	Motility test	D-Glucose	Gas production	indole	Probable organism
LAB 1	+	-	-	-	+	-	-	*L. plantarum*
LAB 2	+	-	-	-	-	-	-	*L.paracasei*

Key: + = Positive and - = Negative.

4.1.2 *Lactobacillus plantarum* colonial morphology

After performing MRS culture medium, the strain showed in Plate III shows neat white, round, edge, centrally slightly raised, a diameter of about 1.6 mm, the surface is thick and opaque, smooth.

Plate III. Lactic Acid Bacteria isolate on deManRogosa Sharpe (MRS) agar

4.1.3 PCR Amplification of the 16s rRNA Region of the Lactic Acid Bacterial Isolates

Gel electrophoresis of PCR products is shown in Plate IV which verified the specific amplification of target sequence and comparison to Genebank database led to identification of *Lactobacillus plantarum* and *Lactobacillus paracasei* as they appear on the 1500bp region.

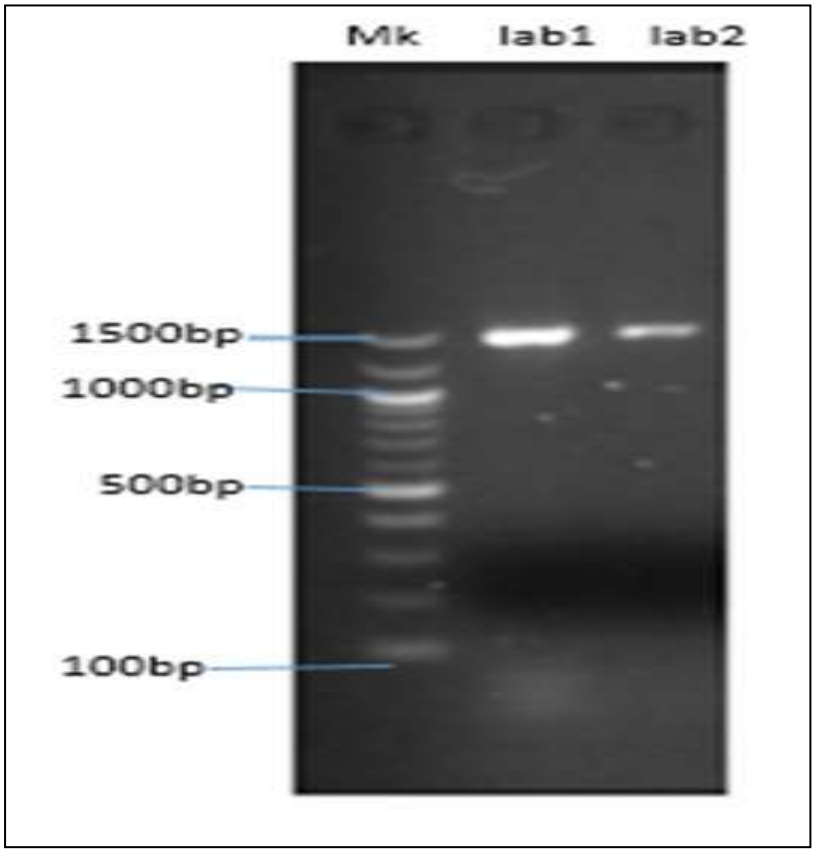

Plate IV. Agarose gel electrophoresis showing the positive amplification of the 16s RNA region of the selected bacteria isolates

4.1.3.1 *Blast Analysis showing the relationship between isolate sequence and the most closely related as present in the NCBI Data Base.*

Table 4.3 depict the results of basic local alignment search tool (BLAST) for Denaturing gradient gel electrophoresis (DGGE) bands excised of the lactic acid bacterial isolates isolated from fresh cow milk identified as *Lactobacillus plantarum* and *Lactobacillus paracasei*

Table 4.3 Blast Analysis showing the Relationship between Isolate sequence and the most closely related as Present in the NCBI Data Base

sample ID	Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
1	*Lactobacillus plantarum*	2806	14001	100%	0	99.87%	AP019815.1
2	*Lactobacillus paracasei*	2747	13705	99%	0	99.80%	CP039707.1

Source: International Institute of Tropical Agriculture Ibadan.

4.1.4 Physico-chemical parameters of fresh juices

4.1.4.1 *Physico-chemical characteristics of fresh orange juice*

Physico-chemical characteristics of fresh orange juice (Table 4.4) showed that it is a good source of total soluble solids (TSS) (4.69±0.027 °B), reducing sugar (3.89±0.102 %), titratable acidity (TTA) (0.027±0.001 %) and pH (5.92±0.070). This similar observations have been reported earlier for these physico-chemical characteristics of orange juice by; (Leahu *et al*., 2011; Shukla and Kushwaha, 2017 and Cristiny *et al*., 2020).

4.1.4.2 *Physico-chemical characteristics of fresh watermelon juice*

Table 4.4 showed that it is a good source of total soluble solids (TSS) (7.76±0.033 ^{0}B), reducing sugars (4.55±0.024 %), titratable acidity (TTA) (0.028±0.001 %) while, pH value of the juice was recorded as 5.20±0.06. Similar observations have been reported earlier for

these physico-chemical characteristics of watermelon juice by (Okereke *et al*., 2016; Fernandes Pereira and Rodrigues, 2018). A perusal of the entire data (Table 4.4) showed that the two fruits juices could be considered as suitable for lactic acid fermentation.

Characteristics	**Orange**	**Watermelon**
pH	5.92±0.07	5.20±0.06
TTA	0.027±0.001	0.028±0.001
Reducing sugar	3.89±0.102	4.55±0.024
TSS	4.69±0.027	7.76±0.033

Table 4.4: Physico-chemical characteristics of the fresh fruits juice (orange and watermelon)

Keys: TTA= Total titratable acid, TSS = Total soluble solids, pH = power of Hydrogen ion

4.1.5 Physiochemical and microbiological analysis of the probiotic juice inoculated with probiotic bacteria during fermentation.

4.1.5.1 *Physio-chemical and microbiological analysis of orange juice inoculated with probiotic bacteria during lactic acid fermentation.*

At the end of 72 h of fermentation, Table 4.5 reveal that there is a significant difference between the treated fruit juice with probiotic bacteria and the control sample in the reducing sugar, the antimicrobial activity of the orange probiotic fruit juice also shows that there is a significant difference between the treated fruit juice (T1, T2 and T3) and the control fruit juice sample (T4) at $p \leq 0.5$ level of significance, Titratable acidity (TTA) and specific gravity (SG) has no significant difference between the probiotic fruit juice and the control. The pH, viable cell counts, Total soluble solid and lactic acid bacteria count has significant difference between the probiotic fruit juice (T1, T2 and T3) and the control fruit juice (T4) $p \leq 0.5$ level of significance

Table 4.5 Physiochemical and Microbiological analysis of orange Juice inoculated with Probiotic Bacteria during Fermentation

Parameters	**T_1**	**T_2**	**T_3**	**T4**
Reducing sugars (%)	3.23 ± 0.33^a	3.023 ± 0.35^a	3.10 ± 0.39^a	3.54 ± 0.26^b
Antimicrobial activity	21.25 ± 4.45^b	20.83 ± 4.36^b	22.75 ± 5.02^b	8.00 ± 0.00^c
TTA	0.029 ± 0.003^a	0.035 ± 0.003^a	0.041 ± 0.004^a	0.034 ± 0.002^a
Ph	5.20 ± 0.39^a	5.18 ± 0.33^a	4.90 ± 0.35^a	5.45 ± 0.35^b
Viable cell counts	4.85 ± 1.32^a	4.05 ± 1.06^a	5.36 ± 1.41^a	4.33 ± 1.53^b
Total soluble solids(°B)	4.34 ± 0.08^a	4.48 ± 0.14^a	4.46 ± 0.12^a	4.87 ± 0.05^b
SG	1.02 ± 0.0030^a	1.02 ± 0.0009^a	1.02 ± 0.0009^a	1.04 ± 0.001^a
Lactic Acid count	2.90 ± 1.21^a	2.42 ± 1.05^a	3.37 ± 1.43^a	0.00 ± 0.00^b

Means with different superscript in the same row are significantly different ($p < 0.05$) for the parameter measured, but means with same superscripts are not significantly different ($p > 0.05$).

Keys: ± (SE); T_1 (*L. plantarum* 10^6 CFU/mL); T_2 (L. *paracasei* 10^6 CFU/mL); T_3 (*L. plantarum and L.paracasei* 10^6 CFU/mL); T4= Control

4.1.6 Physiochemical and microbiological analysis of watermelon inoculated with probiotic bacteria during lactic acid fermentation.

At the end of the 72 h fermentation Table 4.6 reveal that there is a significant difference between the treated fruit juice with probiotic bacteria and the control sample in the reducing sugar, the antimicrobial activity of the watermelon probiotic fruit juice also shows that there is a significant difference between the treated fruit juice (T1, T2 and T3) and the control fruit juice sample (T4) at $p \leq 0.5$ level of significance, Titratable acidity (TTA) and specific gravity (SG) has no significant difference between the probiotic fruit juice and the control. The pH, viable cell counts, Total soluble solid and lactic acid bacteria count has significant difference between the probiotic fruit juice (T1, T2 and T3) and the control fruit juice (T4) $p \leq 0.5$ level of significance.

Table 4.6 Physiochemical and Microbiological Analysis of Watermelon inoculated with Probiotic Bacteria during Fermentation

Parameters	T1	T2	T3	T4
Reducing sugars (%)	3.93 ± 0.28^{ab}	4.00 ± 0.26^{ab}	3.53 ± 0.48^{a}	4.22 ± 0.12^{c}
Antimicrobial activity	21.10 ± 4.41^{c}	21.13 ± 4.44^{c}	23.13 ± 5.11^{b}	8.00 ± 0.00^{f}
TTA	0.028 ± 0.002^{a}	0.032 ± 0.003^{a}	0.039 ± 0.004^{a}	0.034 ± 0.002^{a}
pH	4.62 ± 0.25^{ab}	4.66 ± 0.22^{ab}	4.39 ± 0.29^{a}	5.08 ± 0.06^{d}
Viable cell counts	4.43 ± 1.21^{ab}	4.18 ± 1.13^{ab}	5.48 ± 1.47^{a}	1.05 ± 0.12^{b}
Total soluble solids (°B)	6.69 ± 0.41^{b}	6.73 ± 0.40^{b}	6.69 ± 0.55^{a}	7.60 ± 0.08^{e}
SG	1.03 ± 0.006^{a}	1.024 ± 0.006^{a}	1.025 ± 0.005^{a}	1.04 ± 0.001^{b}
Lactic Acid count	2.70 ± 1.09^{a}	2.55 ± 1.07^{a}	3.07 ± 0.005^{a}	0.00 ± 0.00^{b}

Keys: ± (SE); T_1 (*L. plantarum* 10^6 CFU/mL); T_2 (L. *paracasei*10^6 CFU/mL); T_3 (*L. plantarum andL.paracasei*10^6 CFU/mL); T4= Control. SG = specific gravity, CFU/mL = Colony Forming Unit/Milliliter.

Means with different superscript in the same row are significantly different ($p < 0.05$) for the parameter measured, but means with same superscripts are not significantly different ($p > 0.05$).

4.1.7 Physico-chemical and microbiological characteristics of probiotic fruits juices during cold storage (4 °C)

4.1.7.1 *Effect of cold storage on physico-chemical parameters of orange and watermelon probiotic juice after completion of lactic acid fermentation*.

Selection of the best treatment from each of the probiotic juice was used on the basis of their physico-chemical and microbiological parameters that was obtained during the fermentation days. The third treatment (*L. plantarum* and *L. paracasei*) (T3 consortium) was used for cold storage studies.

The trend of change in pH during cold storage (4 °C) of probiotic watermelon juice is depicted in Table 4.7 in which pH increased with increase time(weeks) of storage. The table reveals the effect of cold storage (4 °C) on the pH value of watermelon probiotic juice. Initially, pH value of watermelon probiotic juice was 3.85±0.01 during storage, the pH ranged from 3.85±0.01-3.92±0.01. The storage interval (weekly) has a significant effect on pH value of watermelon probiotic juice.

The trend of change in titratable acidity during cold storage (4 °C) of probiotic watermelon juice is depicted in Table 4.7, in which titratable acidity is decreasing with increase in time interval (weeks) of storage. The table reveals the effect of cold storage (4 °C) on titratable acidity of watermelon probiotic juice. Initially, titratable acidity value of orange probiotic juice was 0.05±0.00 which during storage decreased to 0.03±0.00. Cold storage interval (weekly) has a significant effect on titratable acidity of watermelon probiotic juice.

The trend of change in TSS during cold storage (4 °C) of probiotic watermelon juice is in which TSS is decreased with increase in time (0- 4weeks) of storage. The table summarizes

the effect of cold storage (4 °C) on TSS of watermelon probiotic juice. Initially, TSS value of watermelon probiotic juice was 5.16±0.00 °B and during storage, TSS ranged from 5.16±0.00–5.02±0.01 °B. The storage interval (weekly) had a significant effect on TSS of watermelon probiotic juice.

The trend of change in reducing sugar during cold storage (4 °C) of probiotic watermelon juice is shown in Table 4.7 in which reducing sugar is decreased with increase of time (weeks) of storage. Initially, reducing sugar value of watermelon probiotic juice was 2.83±0.01 % which during storage decreased to 2.18±0.001 %. Cold storage interval (weekly) has a significant effect on reducing sugars value of watermelon probiotic juice.

Table 4.7: Effect of Cold Storage (4 ºC) on physico-chemical parameters of watermelon juice.

Parameters \Time interval	0 week	1 week	2 weeks	3 weeks	4 weeks
pH	3.86±0.01[c]	3.88±0.00[c]	3.94±0.00[c]	3.96±0.00[c]	3.98±0.01[c]
TTA	0.05±0.00[a]	0.04±0.00[a]	0.04±0.00[a]	0.03±0.000[a]	0.03±0.00[a]
TSS	5.16±0.00[d]	5.12±0.01[d]	5.07±0.00[d]	5.04±0.0[d]	5.02±0.01d[d]
Reducing sugar	2.83±0.01[b]	2.24±0.00[b]	2.21±0.00[b]	2.27±0.003[b]	2.18±0.001[b]

Keys: TTA =Titratable acid, TSS =Total soluble acid.

Means with different superscript in the same row are significantly different ($p < 0.05$) for the parameter measured, but means with same superscripts are not significantly different ($p > 0.05$).

4.1.8 Effect of Cold Storage (4 ºC) on physico-chemical parameters of orange juice

The trend of change in pH during cold storage (4 ºC) of probiotic orange juice is depicted in Table 4.8 in which pH increased with increase time(weeks) of storage. The table reveals the effect of cold storage (4 ºC) on the pH value of orange probiotic juice. Initially, pH value of watermelon probiotic juice was 3.63±0.033 during storage, the pH ranged from 3.63±0.033-3.73±0.006. The storage interval (weekly) has a significant effect on pH value of orange probiotic juice.

The trend of change in titratable acidity during cold storage (4 °C) of probiotic orange juice is depicted in table 4.8, in which titratable acidity is decreasing with increase of time interval (weeks) of storage. The table reveals the effect of cold storage (4 °C) on titratable acidity of orange probiotic juice. Initially, titratable acidity value of orange probiotic juice was 0.05±0.003 % which during storage decreased to 0.03±0.003 %. Cold storage interval (weekly) has a significant effect on titratable acidity of orange probiotic juice. The trend of change in TSS during cold storage (4 ºC) of probiotic orange juice is shown in figure 4.8 in which TSS is decreased with increase in time (0-4weeks) of storage. The table summarizes the effect of cold storage (4 ºC) on TSS of orange probiotic juice. Initially, TSS value of orange probiotic juice was 4.68±0.006 °B and during storage, TSS ranged from 4.68±0.006-4.45±0.003 °B. The storage interval (weekly) had a significant effect on TSS of orange probiotic juice.

The trend of change in reducing sugar during cold storage (4 °C) of probiotic Orange juice is shown in Table 4.8 in which reducing sugar is decreased with increase of time (weeks) of storage. Initially, reducing sugar value of Orange probiotic juice was 2.20±0.003 % which during storage decreased to 2.14±0.003 %. Cold storage interval (weekly) has a significant effect on reducing sugars value of Orange probiotic juice.

Table 4.8: Effect of cold storage (4 °c) on physico-chemical parameters of orange juice.

Parameters \Time interval	0 week	1 week	2week	3week	4week
pH	3.63±0.033[a]	3.66±0.003[a]	3.68±0.003[a]	3.72±0.003[b]	3.73±0.006[b]
TTA	0.05±0.003[c]	0.04±0.000[c]	0.04±0.001[c]	0.03±0.000[c]	0.03±0.003[c]
TSS	4.68±0.006[d]	4.63±0.003[d]	4.53±0.030[d]	4.48±0.006[d]	4.45±0.003[d]
Reducing Sugar	2.20±0.003[b]	2.19±0.003[b]	2.17±0.003[b]	2.16±0.003[b]	2.14±0.003[b]

Keys: TTA =Titratable acid, TSS =Total soluble acid,

Means with different superscript in the same row are significantly different ($p < 0.05$) for the parameter measured, but means with same superscripts are not significantly different ($p > 0.05$).

4.1.9 Antimicrobial activity of probiotic orange juice

The trend of change in antimicrobial activity during cold storage (4 ºC) of probiotic orange juice is shown in Figure 4.1. The antimicrobial activity is decreasing with increase in time (weeks) of the storage. The figure summarizes the effect of cold storage (4 ºC) on antimicrobial activity against *Escherichia coli* of orange probiotic juice. Initially, antimicrobial activity of orange probiotic juice against *Escherichia coli* was 20mm, during storage, antimicrobial activity against *Escherichia coli* ranged from 20-12 mm. The line graph revealed that antimicrobial activity against *E.coli* is decreased with increase of time (weeks). Cold storage interval (weekly) has a significant effect on antimicrobial activity of orange probiotic juice.

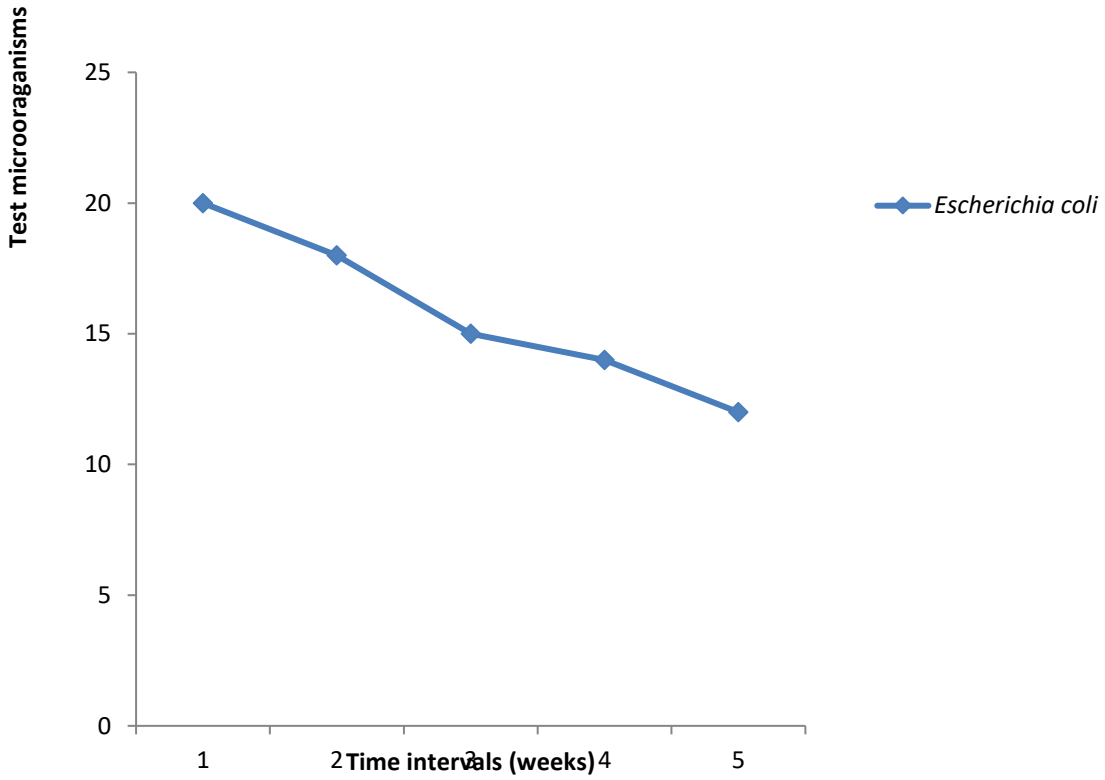

Figure 4.1: Zone of Inhibition (mm) of the Test Organism (*Escherichia coli*) on Probiotic Orange Juice

4.1.10 Effect of cold storage (4 ºC) on microbiological quality of orange probiotic juice

The trend of change in viable cell counts during cold storage (4 °C) of probiotic orange juice is shown in Table 4.9 in which viable cell counts are decreased with increase in time (weeks) of storage. The initial viable cell count of orange probiotic juice is 3.65±0.05 CFU/mL. During storage, the viable cell counts ranged from 3.65±0.05-2.00±0.00 CFU/mL. Data revealed that viable cell counts are decreasing with increase in time (weeks). Cold storage interval (weekly) had a significant effect on viable cell counts of orange probiotic juice.

The trend of change in lactic acid bacteria counts during cold storage (4 ºC) of the orange probiotic juice is shown in Table 4.9, in which lactic acid bacteria counts are decreasing with increase in time(weeks) of storage**.** The Initial lactic acid bacteria count of orange probiotic juice is 3.20±0.00 CFU/mL. During storage, the lactic acid bacteria counts ranged from 3.20±0.00-0.80±0.00 (CFU/mL). Data revealed that lactic acid bacteria counts are decreasing with increase in time (weeks). Cold storage interval (weekly) had a significant effect on lactic acid bacteria counts of orange probiotic juice.

Table 4.9 revealed the effect of cold storage (4 ºC) on yeast counts of orange probiotic juice. The Initial yeast count of orange probiotic juice is 0.00±0.00. During storage, the yeast counts ranged from 0.00±0.00-0.46±0.005 (CFU/mL). Data revealed that yeast counts are increasing with increase in time (weeks). Cold storage interval (weekly) had a significant effect on yeast counts of orange probiotic juice.

Table 4.9: **Effect of cold storage (4 ºC) on microbiological quality of orange probiotic juice**

Parameters\Time interval	0 week	1 week	2 week	3 week	4 week
Lactic Acid Bacteria ($1x10^9$ CFU/mL)	3.20 ± 0.00^{c}	3.01 ± 0.00^{c}	2.55 ± 0.05^{c}	1.35 ± 0.05^{c}	0.80 ± 0.00^{c}
Viable cell count ($1x10^9$ CFU/mL)	3.65 ± 0.05^{b}	3.50 ± 0.00^{b}	3.25 ± 0.05^{b}	2.80 ± 0.000^{b}	2.00 ± 0.00^{b}
Yeast cells ($1x10^9$ CFU/mL)	0.00 ± 0.00^{a}	0.66 ± 0.005^{a}	0.25 ± 0.000^{a}	0.36 ± 0.005^{a}	0.46 ± 0.005^{a}

Means with different superscript in the same row are significantly different ($p < 0.05$) for the parameter measured, but means with same superscripts are not significantly different ($p > 0.05$).

4.1.11 Effect of cold storage (4 ºC) on microbiological quality of Watermelon Probiotic Fruit Juice

The trend of change in viable cell counts during cold storage (4 °C) of probiotic watermelon juice is shown in Table 4.10 in which viable cell counts are decreased with increase in time (weeks) of storage. The Initial viable cell count of watermelon probiotic juice is 3.95±0.05 CFU/mL. During storage, the viable cell counts ranged from 3.95±0.05-2.50±0.00 CFU/mL. Data revealed that viable cell counts are decreasing with increase in time (weeks). Cold storage interval (weekly) had a significant effect on viable cell counts of watermelon probiotic juice.

The trend of change in lactic acid bacteria counts during cold storage (4 °C) of the watermelon probiotic juice is shown in Table 4.10, in which lactic acid bacteria counts are decreasing with increase in time (weeks) of storage .The Initial lactic acid bacteria counts of watermelon probiotic juice is 3.45±0.15 CFU/mL. During storage, the lactic acid bacteria counts ranged from 3.45±0.15 CFU/mL - 1.00±0.00 (CFU/mL). Data revealed that lactic acid bacteria counts are decreasing with increase in time (weeks). Cold storage interval (weekly) had a significant effect on lactic acid bacteria counts of watermelon probiotic juice.

Table 4.10 revealed the effect of cold storage (4 °C) on yeast counts of watermelon probiotic juice. The initial yeast count of watermelon probiotic juice is 0.00±0.00. During storage, the yeast counts ranged from 0.00±0.00-0.50±0.000 (1×10^9 CFU/mL). Data revealed that yeast counts are increasing with increase in time (weeks). Cold storage interval (weekly) had a significant effect on yeast counts of watermelon probiotic juice.

Table 4.10: Effect of cold storage (4 ºC) on microbiological quality of watermelon probiotic juice

Parameters\Time interval	0 week	1 week	2week	3week	4week
Lactic Acid Bacteria ($1x10^9$ CFU/mL)	3.45 ± 0.15^{a}	2.90 ± 0.00^{b}	2.45 ± 0.05^{a}	1.20 ± 0.00^{a}	1.00 ± 0.00^{a}
Viable cell count ($1x10^9$ CFU/mL)	3.95 ± 0.05^{c}	3.30 ± 0.00^{c}	3.10 ± 0.00^{c}	2.85 ± 0.05^{c}	2.50 ± 0.00^{c}
Yeast cells ($1x10^9$ CFU/mL)	0.00 ± 0.00^{b}	0.68 ± 0.005^{b}	0.33 ± 0.005^{b}	0.40 ± 0.000^{b}	0.50 ± 0.000^{b}

Means with different superscript in the same row are significantly different ($p < 0.05$) for the parameter measured, but means with same superscripts are not significantly different ($p > 0.05$).

4.1.12 Sensory profile of the orange and watermelon probiotic fruit juice

Sensory profiles of watermelon and orange probiotic juice produced by fermentation with of *L. plantarum* , *L paracasei* and their mixed culture are presented in Figure 4.2 and 4.3 respectively. The sensory evaluation of the mixed culture *L. plantarum* and *L. paracasei* (T3) shows higher acceptability than the fruit juice inoculated with the single strain (T1 and T2) *L. plantarum* and *L. paracasei* respectively.

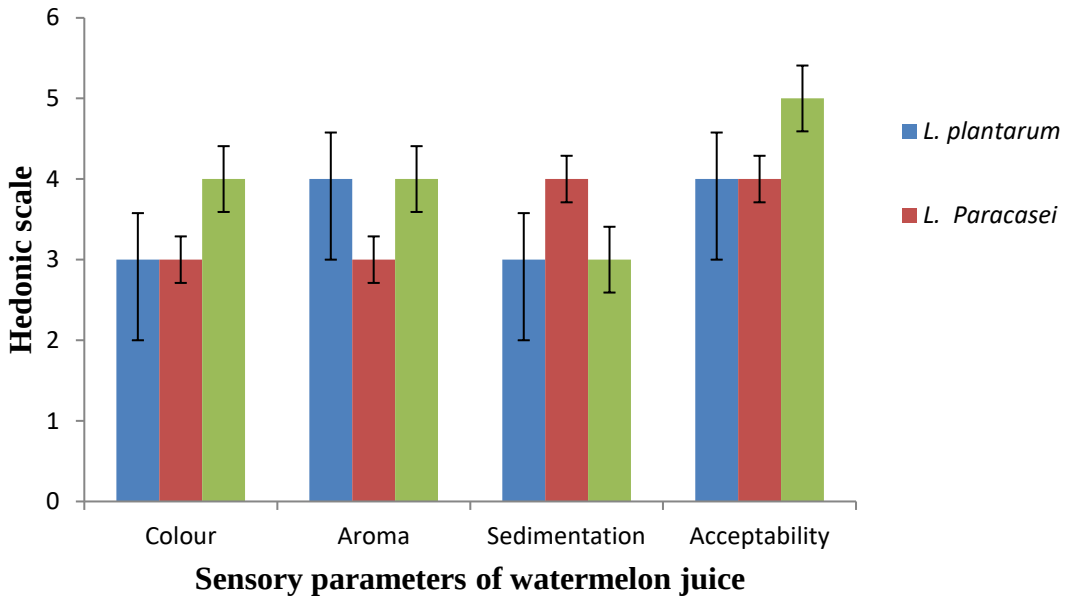

Figure 4.2: sensory parameters of watermelon probiotic juice

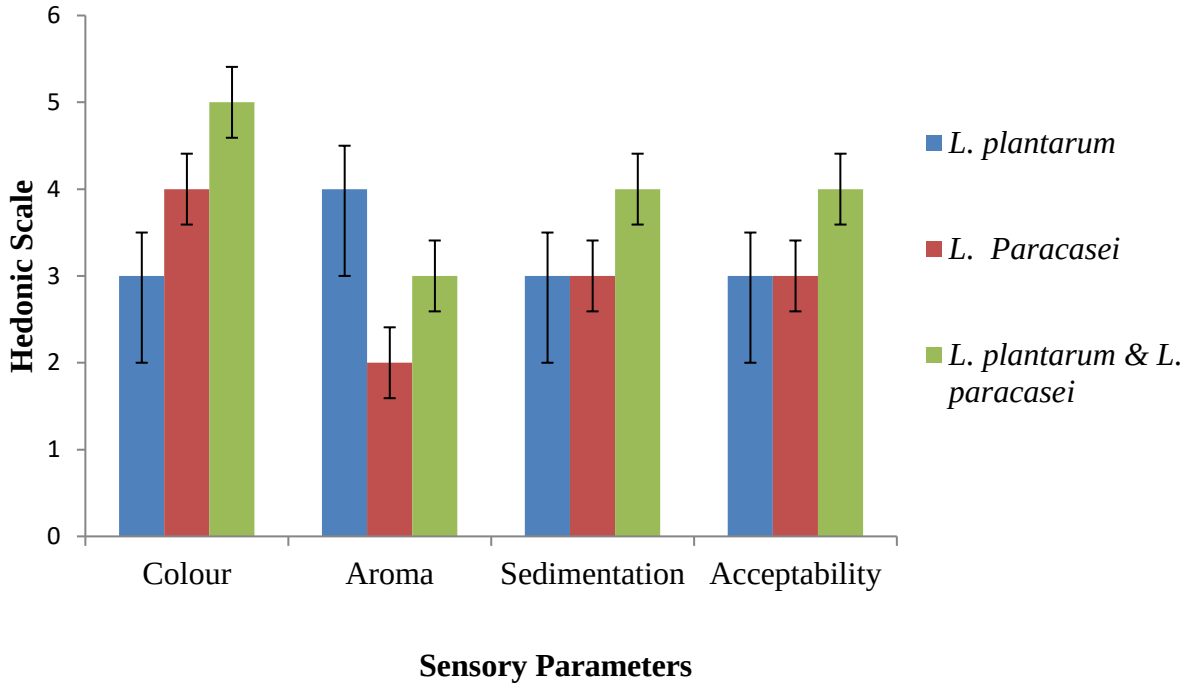

Figure 4.3: Sensory Parameters of Orange Probiotic Juice

4.2 Discussion

The physico-chemical properties of the fruit juices were measured before and after probiotication. The acidity of the substrates increased during probiotication, physico-chemical properties of the freshly prepared fruit juices were of the following values, The pH were 5.92±0.07 and 5.20±0.6 for orange and watermelon juice respectively, with percentage titrable acidity of 0.027±0.001 and 0.028±0.001, reducing sugar (%) 3.89±0.012 and 4.55±0.024, total soluble solid (°B) 4.69±0.27 and 7.76±0.033 for orange and watermelon respectively in all the parameters as seen in Table 4.4. This result is in accordance with the work done by Okereke *et al*. (2016) on physico- chemical analysis of fresh fruit juices. The Physico-chemical analysis of fermented orange and watermelon probiotic fruit juices at different time intervals (0, 24, 48 and 72 h) revealed that the parameters analysed decreases in values as the time increases except for the titrable acidity (%) which increased progressively. However, the reducing sugar decreased due to utilization of the sugar for the growth of the bacteria as seen in Table 4.5 and 4.6. The pH decreases from 4.3-3.8 and 5.2-4.1, while the titrable acidity increases from 0.03±0.01-0.05±0.01, 0.06±0.01-0.09±0.01, for orange and watermelon respectively. A rapid decrease in pH in the beginning of fermentation is of great importance for the quality of the end product, this agrees with the work of (Worku *et al*., 2019). The rapid increase in acidity minimizes the influence of spoilage bacteria in the slowly acidified medium. The changes in pH and acidity during fruit juices fermentation by *L. plantarum,* (T1), *L. paracasei* (T2) and their mixture (consortium) T3 in both orange and watermelon is significantly different from the control sample (T4) that was not inoculated with lactic acid bacteria. The lactic acid mixed culture (consortium) *L. plantarum* and *L. paracasei* (T3) showed a more rapid

decrease in pH (4.90±0.35), (4.39 ± 0.29) for orange and watermelon probiotic fruit juice respectively than the other two fruit juice cultures examined 5.20±0.39 and 5.18±0.33 for T3 and T2 respectively for orange probiotic juice after fermentation in Table 4.5 and 4.6 However, this result agrees with that of (White and Hekmat, 2018) who studied the probiotication of Apple cider and grape juice by lactic acid bacteria and found out that the lactic acid cultures reduced the pH to 4.1 or below and increased the acidity to 0.65 % or higher, and the viable cell counts (CFU) reached nearly 1.0 to 9.0×10^{9}/ mL after 72 hours of fermentation. The lactic acid cultures rapidly fermented the juices and reduced the level of sugar.

Reducing sugar content and total soluble solid (TSS) were decreased with time. It is anticipated that bacteria cells used the sugar and total soluble solid as food. As a result, reducing sugar and TSS content were decreased gradually from 4.49-2.25 and 4.12-3.25 respectively for the consortium of watermelon probiotic juice. This may be due to bacteria cell was capable of growing in fruit juice without additional nutrient supplement.

The acidity of the fermented samples during storage period was low and varied a little, among different weeks; (0.05±0.003 - 0.03±0.003) as seen in Table 4.8, This acidic condition could lead to the decrease in the maximum growth rate and an extended length of the lag phase. During this period metabolic activity and lactic acid production is minor and the bacteria cells tried to adapt with the new conditions. Reducing sugar content 2.20±0.003 and total soluble solid (TSS) 4.68±0.006 were decreased with time to 2.14±0.003 and 4.45±0.003 respectively in table 4.8. It is anticipated that bacteria cells used the available sugars and total soluble solid as food. As a result, reducing sugar and TSS content were

decreased gradually, this may also be due to bacteria cell was capable of growing in fruit juice without additional nutrient supplement.

Microbial viability is the most important factor during storage period. In this study, *Lactobacillus plantarum* strain MerLAB1 and *Lactobacillus paracasei* strain MerLAB2 culture viability decreases with time down the 4weeks during storage at 4 ºC as shown in Table 4.9 and 4.10, at 0 week the lactic acid bacteria count was 3.20±0.00 and decreased to 0.80±0.00 at 4 weeks of orange probiotic juice, this could be as a result of depletion of nutrient. The results indicated that both *Lactobacillus plantarum* strain MerLAB1and *Lactobacillus paracasei* strain MerLAB2 culture were able to withstand and utilized fruit juices for their cell synthesis, as indicated by a decrease in fruit sugar and an increase in acidity. After one week, *Lactobacillus* population started to reduce. Juice inoculated with the mixed culture T3 showed the best performance in terms of population density than other samples. The total count of *Lactobacillu*s for the probiotic orange and watermelon juice samples were well above the standard value (10^7) up to day 14. This high viable count is important for maximum health benefits by probiotic foods (Thakur and Joshi, 2017). This result agrees with the report of Hossain *et al*. (2020) who stated that probiotic pineapple juice could be considered as a probiotic beverage without any nutritional supplement. The growth of these probiotic bacteria could have been affected by various factors such as availability of oxygen, fermentation time and storage temperature (Martins *et al*., 2013). It has been also reported that the acid production ability of lactic acid bacteria, especially post incubation (post-acidification) affects the cell viability of probiotic bacteria, including *L. acidophilus and Bifidobacterium bifidum* (Hossain *et al*., 2020).

L. plantarum and *L. paracasie* mixed culture (T3) probiotic juice samples had the best antagonistic activity against the test pathogenic *Escherichia coli* with the activity of (30mm) after 72 h of fermentation of the orange juice *L. plantarum* had the least zone of inhibition of (27mm) against the test organism *Escherichia coli*. The three fruit juice samples (T1 T2and T3) had antagonistic activity against the pathogenic test organism with zones of inhibitions.

Effect of cold storage on physico-chemical parameters and cell viability of probiotic fruits juices. During cold storage (4 °C), physico-chemical parameters such titratable acidity, total soluble solids, reducing sugar, and antimicrobial activity against pathogenic bacteria *E. coli* slightly decreased, while pH is also slightly increased in both the orange and watermelon probiotic juice. Viability of lactic acid bacteria counts of the probiotic fruits juices are also slightly decreased during cold storage (0-4 weeks) due to lack of ability to survive in the stressful condition of low pH and high acidity. The reduction in the TSS during fermentation and storage may be due to the utilization of sugars and other metabolic activity by the probiotic LAB in the probiotic juice samples. This agrees with the report of Tayo and Akpeji, (2016), who observed a similar reduction in fruit juice with (*Pediococcus pentosaceus* LaG1, *Lactobacillus rhamnosus* GG, *Pediococcus pentosaceus* LBF2) on pineapple juice.

The sensory evaluation of the probiotic fruits juice (watermelon and orange) was done by using descriptive analysis without tasting. Colour, aroma, sedimentation and acceptability were evaluated; these characteristics were scored out of 5. Sensory evaluation of the probiotic fruits juices concluded that, lactic acid fermentation did not influence the sensory characteristics of fruit. Most of the sensory characteristics remained desirable. The results

of hedonic tests reflect that the Colour, aroma, sedimentation and overall acceptability of probiotic orange and watermelon juice were preferred by the panelists. From the results it is apparent that lactic acid fermentation retained many of the desirable characteristics. Thakur, (2015) found similar results in case of mango and pineapple probiotic juice.

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Lactobacillus plantarum strain MerLAB1 and *Lactobacillus paracasei* strain MerLAB2 were successfully isolated from fresh cow milk, their presence in fresh cow milk is of great benefits to humans and animals either as supplements or food production processes.

Watermelon and orange probiotic fruit juices were produced using lactic acid fermentation, *Lactobacillus plantarum* strain MerLAB1 and *Lactobacillus paracasei* strain MerLAB2 were used to produce probiotic orange and watermelon juice, and their ability to produce lactic acid and cell synthesis were checked, both of them were grown in these juices at 37 °C and the viable counts reached over 10^6 CFU/mL after 48 hours of fermentation at 37 °C. The results of the present study demonstrated that both *L. plantarum* and *L. paracasei* were able to survive in fermented juices with high acidity.

The physico-chemical properties such as, Titratable Acidity, Total Soluble Solids, Specific Gravity, pH and Reducing Sugar were determined at different time intervals (0, 24, 48 and 72 h) of the probiotic fruit juice during lactic acid fermentation at 37 ºC.

During storage, the cell viability were viable throughout the storage period at 4 ºC for 4weeks.The cell reached 10^9 CFU/mL but were slightly reduced when compared with the fermentation days. Their physico-chemical parameters were checked on weekly basis. The fermented probiotic and stored juice had antagonistic effect on the enteric pathogenic *Escherichia coli*.

The sensory properties such as Color, Aroma, Sedimentation and Acceptability were evaluated by 10 panelists. The results obtained in this study will be useful for the development of probiotic fruit juice with health beneficial effects. This could serve as a nutraceutical with health benefits for vegetarians, lactose intolerant people, and those who are allergic to milk products. Probiotic *Lactobacillus plantarum* strain MerLAB1 and *Lactobacillus paracasei* strain MerLAB2 is suggested for development of probiotic orange and watermelon juice.

5.2 Recommendations

1. More extensive in *vitro* and in *vivo* studies are vital in order to authenticate the probiotic potential and safety of such cultures and fruit products based on these beneficial microbes before being endorsed for the better health and nutrition of society.

2. Further work is necessary to check the biological evaluation of the products and the production of probiotic juice using more Lactic Acid Bacteria (LAB) consortium.

3. New studies should be carried out to produce several new probiotic beverages combining dairy and non-dairy substrates. This may result in new products with the accepted taste and flavor of dairy products and the numerous nutrients of non-dairy products such as fruit juices, vegetables, and cereals.

4. New studies should be focused in traditional non-dairy beverages around the world, in order to identify new potential probiotic microorganisms and new ingredients as potential substrates. Research is needed in order to overcome several problems with the survival of probiotic microorganisms under the harsh conditions of non-dairy substrates, for example, the low pH of fruit juices.

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Appendix

Pairwise genetic distance between isolates and selected identified spp (sequence generated from NCBI data base)

sample ID	LAB1	LAB2
LAB1		
LAB2	0.054	
NR_044704.2_*Lactobacillus_brevis*	0.039	0.055
NR_043182.1_*Lactobacillus_acidophilus*	0.086	0.083
NR_029106.1_*Lactobacillus_delbrueckii_*	0.082	0.084
NR_042439.1_*Lactobacillus_helveticus*	0.089	0.085
MN453623.1_*Lactobacillus_sp*.	0.000	0.055
AB239468.1_*Lactobacillus_casei_*	0.052	0.002
AB601168.1_*Lactobacillus_plantarum*	**0.000**	0.054
CP039707.1:*Lacticaseibacillus_paracasei*	0.052	**0.001**

>MW543941 *Lactobacillus plantarum* strain MerLAB1

AGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACGAACTCTGGTA
TTGATTGGTGCTTGCATCATGATTTACATTTGAGTGAGTGGCGAACTGGTGAGTAACACGTGGGAAACCGC
CCAGAAGCGGGGGATAACACCTGGAAACAGATGCTAATACCGCATAACAACTTGGACCGCATGGTCCGAG
TTTGAAAGATGGCTTCGGCTATCACTTTTGGATGGTCCCGCGGCGTATTAGCTAGATGGTGGGGTAACGGC
TCACCATGGCAATGATACGTAGCCGACCTGAGAGGGYAATCGGCCACATTGGGACTGAGACACGGCCCAA
ACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGA
GTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTAACTGTTCAGGTA
TTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGC
GTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTC
AACCGAAGAAGTGCATCGGAAACTGGGAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCG
GTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGAG
GCTCGAAAGTATGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCATACCGTAAACGATGAATGCTAAGT
GTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGGCCGCAA
GGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCTACG
CGAAGAACCTTACCAGGTCTTGACATACTATGCAAATCTAAGAGATTAGACGTTCCCTTCGGGGACATGGAT
ACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC
TTATTATCAGTTGCCAGCATTAAGTTGGGCACTCTGGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGG
GGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAACGAGTT
GCGAACTCGCGAGAGTAAGCTAATCTCTTAAAGCCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACA
TGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACC

GCCCGTCACACCATGAGAGTTTGTAACACCCAAAGTCGGTGGGGTAACCTTTTAGGAACCAGCCGCCTAAG
GTGGGACAGATGATTAGGGTGAAGTCGTAACAAGGTA

> MW543942 *Lactobacillus paracasei* strain MerLAB2

GGCGGCGTGCCTAATACATGCAAGTCGAACGAGTTCTCGTTGATGATCGGTGCTTGCACCGAGATTCAACA
TGGAACGAGTGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCTTAAGTGGGGGATAACATTTGGAAA
CAGATGCTAATACCGCATAGATCCAAGAACCGCATGGTTCTTGGCTGAAAGATGGCGTAAGCTATCGCTTTT
GGATGGACCCGCGGCGTATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGATGATACGTAGCCGAAC
TGAGAGGTTGATCGGCCACATTGGGACTGAGACACGGCACAAACTCCTACGGGAGGCAGCAGTAGGGAAT
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TGTTGTTGGAGAAGAATGGTCGGCAGAGTAACTGTTGTCGGCGTGACGGTATCCAACCAGAAAGCCACGG
CTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTTATTGGGCGTAAAGCG
AGCGCAGGCGGTTTTTTAAGTCTGATGTGAAAGCCCTCGGCTTAACCGAGGAAGCGCATCGGAAACTGGG
AAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAAC
ACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGA
TTAGATACCCTGGTAGTCCATGCCGTAAACGATGAATGCTAGGTGTTGGAGGGTTTCCGCCCTTCAGTGCCG
CAGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGG
GCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCT
TTTGATCACCTGAGAGATCAGGTTTCCCCTTCGGGGGCAAAATGACAGGTGGTGCATGGTTGTCGTCAGCT
CGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATGACTAGTTGCCAGCATTTAGTTG
GGCACTCTAGTAAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTT
ATGACCTGGGCTACACACGTGCTACAATGGATGGTACAACGAGTTGCGAGACCGCGAGGTCAAGCTAATCT
CTTAAAGCCATTCTCAGTTCGGACTGTAGGCTGCAACTCGCCTACACGAAGTCGGAATCGCTAGTAATCGCG
GATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAA
CACCCGAAGCCGGTGGCGTAACCCTTTTAGGGAGCGAGCCGTCTAAGGTGGGACAAATGATTAGGGTGAA
GTCGTAACAAGGACT

SENSORY EVALUATION QUESTIONNAIRE

On the study titled

PRODUCTION OF PROBIOTIC FRUIT JUICE (WATERMELON AND ORANGE) USING LACTIC ACID BACTERIA FROM FRESH COW MILK.

Instruction: on the hedonic scale of 5 points (from 1: dislike alot to 5: like alot)

Feedback the grade for the following sensorial qualities of the probiotic juice samples presented.

parameters		1	2	3	4	5
Colour	A					
	B					
	C					
Aroma	A					
	B					
	C					
Sweetness	A					
	B					
	C					
Sourness	A					
	B					
	C					
Acceptability	A					
	B					
	C					

5= like a lot

4= like a little

3= neither like nor dislike

2= dislike a little

1= dislike a lot.

Appendix

Pairwise genetic distance between isolates and selected identified spp(sequence generated from NCBI data base)

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NR_029106.1_*Lactobacillus_delbrueckii_*	0.082	0.084
NR_042439.1_*Lactobacillus_helveticus*	0.089	0.085
MN453623.1_*Lactobacillus_sp.*	0.000	0.055
AB239468.1_*Lactobacillus_casei_*	0.052	0.002
AB601168.1_*Lactobacillus_plantarum*	**0.000**	0.054
CP039707.1:*Lacticaseibacillus_paracasei*	0.052	**0.001**

>MW543941 *Lactobacillus plantarum* strain MerLAB1

AGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAA
GTCGAACGAACTCTGGTATTGATTGGTGCTTGCATCATGATTTACATTTGAGTG
AGTGGCGAACTGGTGAGTAACACGTGGGAAACCGCCCAGAAGCGGGGGATAA
CACCTGGAAACAGATGCTAATACCGCATAACAACTTGGACCGCATGGTCCGAG
TTTGAAAGATGGCTTCGGCTATCACTTTTGGATGGTCCCGCGGCGTATTAGCTA

GATGGTGGGGTAACGGCTCACCATGGCAATGATACGTAGCCGACCTGAGAGGG

YAATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCA

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GAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAG

AGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGT

GCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGC

GTAAAGCGAGCGCAGGCGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTCAAC

CGAAGAAGTGCATCGGAAACTGGGAAACTTGAGTGCAGAAGAGGACAGTGGA

ACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGA

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GAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAG

CGGTGGAGCATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTG

ACATACTATGCAAATCTAAGAGATTAGACGTTCCCTTCGGGGACATGGATACA

GGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGC

AACGAGCGCAACCCTTATTATCAGTTGCCAGCATTAAGTTGGGCACTCTGGTGA

GACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCC

CCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAACGAGTTGCGA

ACTCGCGAGAGTAAGCTAATCTCTTAAAGCCATTCTCAGTTCGGATTGTAGGCT

GCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGCCG

CGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTT

GTAACACCCAAAGTCGGTGGGGTAACCTTTTAGGAACCAGCCGCCTAAGGTGG

GACAGATGATTAGGGTGAAGTCGTAACAAGGTA

> MW543942 *Lactobacillus paracasei* strain MerLAB2

GGCGGCGTGCCTAATACATGCAAGTCGAACGAGTTCTCGTTGATGATCGGTGCT

TGCACCGAGATTCAACATGGAACGAGTGGCGGACGGGTGAGTAACACGTGGGT

AACCTGCCCTTAAGTGGGGGATAACATTTGGAAACAGATGCTAATACCGCATA

GATCCAAGAACCGCATGGTTCTTGGCTGAAAGATGGCGTAAGCTATCGCTTTTG

GATGGACCCGCGGCGTATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGA

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CACAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAGT

CTGATGGAGCAACGCCGCGTGAGTGAAGCCGGCTTTCGGGTCGTAAAACTCTG

TTGTTGGAGAAGAATGGTCGGCAGAGTAACTGTTGTCGGCGTGACGGTATCCA

ACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGG

CAAGCGTTATCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTTTAAGT

CTGATGTGAAAGCCCTCGGCTTAACCGAGGAAGCGCATCGGAAACTGGGAAAC

TTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGA

TATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACGCT

GAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGC

CGTAAACGATGAATGCTAGGTGTTGGAGGGTTTCCGCCCTTCAGTGCCGCAGCT

AACGCATTAAGCATTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAG

GAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAA

CGCGAAGAACCTTACCAGGTCTTGACATCTTTTGATCACCTGAGAGATCAGGTT

TCCCCTTCGGGGGCAAAATGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCG

TGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATGACTAGTTGCCAG

CATTTAGTTGGGCACTCTAGTAAGACTGCCGGTGACAAACCGGAGGAAGGTGG

GGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAA

TGGATGGTACAACGAGTTGCGAGACCGCGAGGTCAAGCTAATCTCTTAAAGCC

ATTCTCAGTTCGGACTGTAGGCTGCAACTCGCCTACACGAAGTCGGAATCGCTA

GTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACAC

CGCCCGTCACACCATGAGAGTTTGTAACACCCGAAGCCGGTGGCGTAACCCTTT

TAGGGAGCGAGCCGTCTAAGGTGGGACAAATGATTAGGGTGAAGTCGTAACAA

GGACT

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	C									
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