CHAPTER ONE

INTRODUCTION

The consumption of fermented milk by man dates from the beginning of civilization, once residues of these products were found in pottery fragments from Neolithic, Bronze and Iron Ages settlements in Britain (McKinley, 2005). It is accepted that the initial consumption of fermented or cultured milk products, such as yoghurt, butter and cheese, occurred around the time as they were recognized as effective means of prolonging the shelf-life of milk (Ross et al., 2002; Tamine and Robinson, 2007). The French called it 'la lait de la vie eternelle' - the milk of eternity as it was believed to have therapeutic powers and gave long life to those who consumed it. The main reasons pointed out for yoghurt consumption is the cultural and the increasing search for healthy foods (McKinley, 2005; Cueva and Aryana, 2008). It is a means of protein intake for an improved healthy living (Cueva and Aryana 2008).

Yoghurt is one of the most traditional cultured milk, which is a product of the lactic acid fermentation of milk by addition of a starter culture containing Streptococcus thermophilus and Lactobacillus delbrueckii subsp. bulgaricus (Steinkraus, 1997; Tamine and Robinson, 2007). Yoghurt is fermented milk, which has its origins in Eastern Europe, but is now widely consumed throughout the world, mainly in northern European countries, Balkans, Middle East and Indian sub-continents (Tamine, 2002). In many of these countries, yoghurt is still manufactured using traditional procedures. Since the last world war, yoghurt consumption has been steadily increasing not only in European countries, but also in the United States, enhancing its industrial-scale production. At present, new types of fermented milk are available,
prepared by adding fruits or flavouring, enriched with vitamins or containing selected intestinal bacteria such as *Lactobacillus acidophilus* and several *Bifidobacterium* species (Kurmann, 1984). In France, the term "yoghurt" can be used legally only to designate the product resulting from milk fermentation brought about exclusively with two thermophilic lactic acid bacteria, *Streptococcus salivarius* subsp. *thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, which must be found alive in the final product (=10 million CFU/g of yoghurt). It is noteworthy that these two conditions are seldom specified by existing legislation in most other yoghurt producing countries (Rybka and Kailasapathy, 1995).

The yoghurt production is quite simple, and the rising consumption of this product has led to the development of automated and sophisticated equipment for the industrial processing. Also, the improved industrial process has not only improved the storage, transportation and commercialization of the product, the microbiological parameters according to Tamine and Robinson (2007) has maintained coliform free products. Despite the practicability, the current processing equipment must provide proper quality and safety (Salinas, 1986). The maintenance of these secure characteristics is essential even in the post-production steps, like storage, transport and commercialization. Microbiological parameters are generally used to verify these conditions, especially by coliforms and lactic acid bacteria (LAB) enumeration (Tamine and Robinson, 2007). At commercial points, yoghurts produced in Brazil must contain at least $10^7$ colony forming units per gram (CFU/g) of LAB and less than 10 most probable number per gram (MPN/g) of thermotolerant coliforms for indicative samples (Rodrigues, 2010). Coliforms quantification allows the verification of raw milk quality and the efficiency of the processing, and LAB enumeration indicates the levels of added starter culture and its development during the
storage and shelf-life. In addition, the measurement of acidity is also observed in order to evaluate the preservation of yoghurt status, varying from 0.6 to 1.5 g of lactic acid per 100 g of product, and the temperature of preservation in dairy industries and markets must not be higher than 10°C (Rodrigues, 2010).

Fermented milk, like the fresh milk from which they are produced, is liable to contamination. Moulds and yeasts are the primary contaminants in yoghurt produced commercially in Nigeria (Oyeleke, 2009). Moulds and yeasts growing in yoghurt utilize some of the acid and produce a corresponding decrease in the acidity, which may favour the growth of putrefactive bacteria (Oyeleke, 2009).

LACTIC ACID BACTERIA (LAB)

Interest in microorganisms as a component of biological diversity has been renewed in recent years. The interest in microorganisms occurring in foods is primarily due to the biotechnological potential of new bacterial species and strains (Leisner et al., 1999). Lactic acid bacteria (LAB) are widely distributed in nature and occur naturally as indigenous microflora in raw milk, drinking yoghurt and other dairy products. They are Gram positive bacteria that play an important role in many food fermentation processes. Some species of the genus Lactobacillus, Lactococcus Leuconostoc and Streptococcus are included in this group. The lactic acid fermentation has long been known and applied by humans for making different food stuffs. For many centuries, LAB have been an effective form of natural preservation. In addition, they strongly determine the flavour, texture and frequently, the nutritional value of food and feed products. However the application of well-studied starter cultures has been established for decades (Lee, 1996).
PROBIOTIC BENEFITS OF YOGHURT

The word ‘probiotic’ is derived from the two Greek words *pro* meaning ‘For’ and *bios* meaning ‘Life’. Probiotic therefore means ‘for life’ (Fuller, 1989). Probiotics are globally defined as live microorganisms, which when consumed or administered in adequate amounts, confer a health benefit on the host (FAO/WHO, 2001).

Interest in the role of probiotics for human health goes back at least as far as 1908 when Metchnikoff suggested that man should consume milk fermented with lactobacilli to prolong life (O'Sullivan, *et al.*, 1992). It is only recently, however, that the interrelationship between intestinal microorganisms and the health benefits derived from it are beginning to be understood. At present, it is generally recognized that an optimum ‘balance’ in microbial population in our digestive tract is associated with good nutrition and health (Rybkaa and Kailasapathy, 1995). The microorganisms primarily associated with this balance are lactobacilli and bifidobacteria. Factors that negatively influence the interaction between intestinal microorganisms, such as stress and diet, lead to detrimental effects in health. Increasing evidence indicates that consumption of ‘probiotic’ microorganisms can help maintain such a favourable microbial profile and results in several therapeutic benefits. In recent years probiotic bacteria have increasingly been incorporated into foods as dietary adjuncts.

**Application of probiotic microorganisms in functional foods**

Consumption of probiotic bacteria via food products is an ideal way to re-establish the intestinal microflora balance. For a culture to be considered a valuable candidate for use as a dietary adjunct and to exert a positive influence, it must conform to certain requirements (Martin and Chou, 1992). The culture must be a normal inhabitant of the human intestinal tract, survives
passage through the upper digestive tract in large numbers, be capable of filling an ecological niche, and have beneficial effects when in the intestine (Gilliland, 1989). In order to survive, the strain must be resistant to bile salts present in the lower intestine, gastric conditions (pH 1–4), enzymes present in the intestine (Lysozyme) and toxic metabolites produced during digestion (Hoier, 1992). The bacteria used in traditional yogurt fermentation, *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, do not belong to the indigenous intestinal flora, are not bile acid resistant and do not survive passage through the gut (Gilliland, 1979). These traditional yogurt bacteria may, nevertheless, have positive effects as a result of fermentation metabolites, either by an inhibitory action towards pathogens or improvement of lactose digestion (Hoier, 1992). Since criteria in literature generally state that not less than a million viable cells/ml probiotic product have to be present for transfer of the ‘probiotic’ effect to consumers (Rybka and Kailasapathy, 1995), it is desirable that the probiotic culture multiply to reach high cell counts in the fermented product and possess a high acid tolerance to ensure high viable cell numbers during storage. The selected strains must be able to ferment milk relatively quickly, either alone or in combination with other strains. The possibility of influencing the composition of the intestinal flora by consuming probiotic bacteria partly depends on the dose level. It is generally recognized that $10^8 – 10^9$ bacteria are necessary at the time of consumption (Speck, 1978). Therefore, the probiotic culture must remain viable in the food carrier up to consumption.
PRODUCTION OF YOGHURT

Yoghurt is produced by the controlled fermentation of milk with bacterial cultures consisting of a mixture of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus*. *L. bulgaricus* are rod with rounded ends shape but *S. thermophilus* has a spherical to ovoid shape with irregular segments. Both are Gram positive, facultative anaerobic, non-motile and non spore-forming bacteria. Successful preparation of yoghurt depends upon the proper symbiotic relationship between the two organisms at equal proportion (Tamime and Robinson, 2007). The sugar in milk (called lactose) is fermented to acid (lactic acid) and it is this that causes the characteristic curd to form. The acid also restricts the growth of food poisoning bacteria and some spoilage bacteria. So, whereas milk is a potential source of food poisoning and only has a shelf life of a few days, yoghurt is safer and can be kept for up to ten days, under proper storage conditions.

There are two major types of yoghurt; set and stirred yoghurt. Set yoghurt (which includes fruit-on-the bottom) is formed in retail pots as lactic acid bacteria ferment lactose into lactic acid giving a continuous gel structure in the consumer container. In stirred yogurt, the acid gel formed during incubation in large fermentation tanks is disrupted by agitation (stirring), and the stirred product is usually pumped through a screen which gives the product a smooth and viscous texture (Tamime and Robinson, 2007). The physical attributes of yoghurts, including the lack of visual whey separation and perceived viscosity, are crucial aspects of the quality and overall sensory consumer acceptance of yoghurts. An understanding of the mechanisms involved in the formation of texture in yoghurts and the impact of processing conditions on texture development may help to improve the quality of yoghurt.
YOGURT MANUFACTURING PROCESS

The main processing steps involved in these two types of yoghurt manufacture include the standardization of milk (fat and protein content), homogenization, milk heat treatment, incubation/fermentation, cooling, and storage.

**Milk Standardization**

Milk is often mixed with skim milk and cream to standardize (or adjust) the fat content to the desired level. Milk powders, including nonfat dry milk, whey protein concentrates, or milk protein concentrate, can be blended with the milk using a powder dispersion unit. The milk solids content (including the fat content) for yoghurt ranges from around 9% for skim milk yoghurt to more than 20% for certain types of concentrated yoghurt. Many commercial yoghurt products have milk solids contents of 14-15% (Tamime and Robinson, 2007). The minimum milk solids non-fat content required in standards or regulations in many countries ranges from 8.2 to 8.6% (Tamime and Robinson, 2007). Codex regulations for yoghurt indicate that the minimum milk protein content is 2.7% (except for concentrated yoghurt where the minimum protein content is 5.6% after concentration) and the maximum fat content is 15% (Codex, 2008). The total solids content of milk can be increased by concentration processes, such as, evaporation under vacuum, and membrane processing (i.e., reverse osmosis and ultra filtration). Stabilizers, such as, pectin or gelatin, are often added to the milk base to enhance or maintain the appropriate yoghurt properties including texture, mouth feel, appearance, viscosity/consistency and to the prevention of whey separation (wheying-off) (Tamime and Robinson, 2007). The use of stabilizers may help in providing a more uniform consistency and lessen batch to batch variation. However, there can be textural defects related to the use of stabilizers, including over-
stabilization and under-stabilization. Overstabilization results in a “jello-like” springy body of yoghurt while a weak “runny” body or whey separation can be produced due to under-stabilization (Vedamuthu, 1991). In some countries, such as, the Netherlands and France, regulations do not allow the use of stabilizers for plain (unsweetened) yoghurt (Tamime and Deeth, 1980). In fruit yoghurts, stabilizers (e.g. pectin) are often added to the fruit preparation to help improve the yoghurt texture.

**Homogenization**

Homogenization of the milk base is an important processing step for yoghurts containing fat. Milk is typically homogenized using pressures of 10-20 and 5 MPa first and second stage pressures, respectively, and at a temperature range between 55 and 65°C. Homogenization results in milk fat globules being disrupted into smaller fat globules and the surface area of homogenized fat globules greatly increases. The use of homogenization prevents fat separation (creaming) during fermentation or storage, reduces whey separation, increases whiteness, and enhances consistency of yoghurts (Vedamuthu, 1991). When milk is homogenized, caseins and whey proteins form the new surface layer of fat globules, which increases the number of possible structure-building components in yoghurt made from homogenized milk (Walstra, 1998). Homogenized milk fat globules act like protein particles due to the presence of protein on the fat surface. Recently, ultra-high pressure homogenization at 200 or 300 MPa was investigated for the production of yoghurt. Compared with a conventional homogenization at 15 MPa, the use of ultrahigh pressure homogenization resulted in an increase in yoghurt firmness and water-holding capacity (Serra et al., 2008, 2009). Ultra-high pressure causes whey protein denaturation as well as partial disruption of the casein micelles.
Heat treatment

Heating of milk is an important processing variable for the preparation of yoghurt since it greatly influences the physical properties and microstructure of yoghurt (Lucey, 2004). In yoghurt manufacture, milk is heated prior to culture addition. The temperature/time combinations for the batch heat treatments that are commonly used in the yoghurt industry include 85°C for 30 min or 90-95°C for 5 min (Tamime and Robinson, 2007). However, very high temperature short time (100°C to 130°C for 4 to 16 s) or ultra-heat temperature (UHT) (140°C for 4 to 16 s) are also sometimes used (Sodini et al., 2004). The heat treatment of milk is also used to destroy unwanted microorganisms, which provides less competition for the starter culture. Yoghurt starter cultures are sensitive to oxygen so heat treatment helps to remove dissolved oxygen assisting starter growth.

Fermentation process

After heat treatment, the milk base is cooled to the incubation temperature used for growth of the starter culture. An optimum temperature of the thermophilic lactic acid bacteria, i.e., Streptococcus subsp. thermophilus and Lactobacillus delbrueckii subsp. bulgaricus, is around 40-45°C. Bacterial fermentation converts lactose into lactic acid, which reduces the pH of milk. During acidification of milk, the pH decreases from 6.7 to ≤4.6. Gelation occurs at pH 5.2 to 5.4 for milk that was given a high heat treatment.

Cooling

When yoghurts have reached the desired pH (e.g., ~4.6), yoghurts are partially cooled (~20°C) before fruit or flavoring ingredients are added. Yoghurt products are often blast chilled to <10°C (e.g., 5°C) in the refrigerated cold store to reduce further acid development (Tamime and
Robinson, 2007). In the production of set yoghurt, yoghurts are directly transferred to a cold store or blast chilled in cooling tunnels. For stirred yoghurts, cooling is first performed by agitation in the jacketed fermentation vat and the product is sheared and smoothened by devices like back-pressure values, high shear devices or sieves.

**BIO-YOGHURT**

One of the most popular dairy products for the delivery of viable *Lactobacillus acidophilus* and *Bifidobacterium bifidum* cells is bio-yoghurt. In recent years, some yoghurt products have been reformulated to include live strains of *L. acidophilus* and species of *Bifidobacterium* (known as AB-cultures) in addition to the conventional yogurt organisms, *S. thermophilus* and *L. bulgaricus*. Bio-yoghurt therefore is yoghurt that contains live probiotic microorganisms, the presence of which may give rise to claimed beneficial health effects.

Adequate number of viable cells, namely the ‘therapeutic minimum’ needs to be consumed regularly for transfer of the ‘probiotic’ effect to consumers. Consumption should be more than 100 g per day of bio-yoghurt containing more than $10^6$ CFU/ml (Rybka and Kailasapathy, 1995). One should aim to consume $10^8$ live probiotic cells per day. Regular consumption of 400–500 g/week of AB-yoghurt, containing $10^6$ viable cells per ml would provide these numbers (Tamime *et al.*, 1995). Survival of these bacteria during shelf life and until consumption is therefore an important consideration.

**Production of AB-yoghurt**

For the production of AB-yoghurt, similar processing procedures to traditional yoghurt are applied with the exception of the incorporation of live probiotic starter cultures. Heat treated, homogenized milk with an increased protein content (3.6–3.8%) is inoculated with the
conventional starter culture at 45°C or 37°C and incubated for 3.5 and 9 hours, respectively (Tamime and Robinson, 2007). The probiotic culture can be added prior to fermentation simultaneously with the conventional yoghurt cultures or after fermentation to the cooled (4°C) product before packaging.

**TYPES OF YOGHURT**

- **Set Yoghurt** - is packed immediately after inoculation and is incubated in the package.
- **Stirred Yoghurt** - after setting, fruit and other condiments can be added with a gentle stirring motion.
- **Drinking Yoghurt** - fruit juice and other flavouring compounds are added and mixed to a thick liquid using high speed mixers.
- **Frozen Yoghurt** - set yoghurt is combined with sugar and stabilizers before being pasteurized and frozen.

The main manufacturing procedures for yoghurt production are represented by the flow chart in figure 1 below.
1. Homogenized whole or low-fat milk

2. Addition of skim milk powder

3. Heat treatment (85-95°C for 30 minutes)

4. Cooled to 40-45°C

5. Starter culture added (2%) at 40-45°C

6. Incubate the mixture at 40-45°C for 4-5 hours

   (Until the pH has fallen to around 4.3)

7. Fermentation stopped by cooling to 4°C

   (Stir the thickened yoghurt)

8. Add fruit, sugar, flavourings, and colourants (if required)

9. Packaging

10. Storage at 4°C

Figure 1: A schematic presentation of the production of yoghurt (Tamime and Robinson, 2007).
LOCATION OF STUDY AREA

This study was conducted in the ancient city of Ilorin, the capital city of Kwara state, Nigeria. Ilorin is one of the largest cities located in the North-central geopolitical zone of Nigeria. Ilorin city is about 450km away from the Federal Capital Territory, Abuja.

OBJECTIVE OF THE STUDY

Ilorin is characterized by low level of environmental sanitation, lack of potable water and poor waste disposal as well as very poor and epileptic power supply which are all needed to maintain the microbiological quality of these yoghurt drinks during storage and marketing. Contamination by coliforms, other bacteria and fungi is therefore probable. Quality indicators in yoghurt include microbial evaluation and pH determination (Tamine and Robinson 2007). This study therefore was aimed at the assessment of the microbiological quality of yoghurt drinks marketed in Ilorin Metropolis of Kwara State in North central Nigeria.
CHAPTER TWO

MATERIALS AND METHODS

2.1 COLLECTION OF SAMPLES

Eight (8) different brands of yoghurt were purchased in duplicates at different locations in Ilorin metropolis and care was taken to ensure that the samples were intact. The eight (8) brands of yoghurt were: Abide, Basako, Hollandia, D-Matrix, Fresh’Yo, Ojoma, Dudu and Savanna. The samples were transported in ice box and stored in the refrigerator prior to use.

2.2 STERILIZATION OF MATERIALS

The work bench was first disinfected by swabbing with cotton wool soaked in 70% alcohol. The glass wares used were washed with detergent, rinsed properly in several changes of tap water and further rinsed with distilled water. The glass wares used included test tubes, pipettes, conical flasks, beakers and McCartney bottles. They were then air dried and wrapped with aluminium foil and sterilized in the hot air oven at 170°C for 2-3 hours. The agar media were dispensed in conical flasks, plugged with cotton wool and wrapped with aluminium foil and thereafter sterilized in the autoclave at 121°C for 15minutes. Aseptic technique was applied in the working environment by ensuring that all work was done near the naked flame of Bunsen burner.

2.3 PREPARATION OF CULTURE MEDIA

Four different kinds of media were used for the isolation of different microorganisms from the samples and they are nutrient Agar (NA), MacConkey Agar, Potato Dextrose Agar (PDA), and De Man Rogosa Sharpe (MRS). Other media used were employed in the various biochemical tests to characterize and identify the bacterial isolates. The biochemical tests media used
included Triple sugar ion agar (TSIA), Nutrient broth, Urease Agar, MR/VP broth, Simmons Citrate agar, etc. Each medium was prepared following the manufactures instructions.

**Preparation of Nutrient Agar (NA)**

Nutrient Agar was used for the isolation of bacteria and total bacterial counts. It was prepared by dissolving 28grams of NA powder in 1000ml of distilled water. The mouth of the conical flask was plugged with cotton wool and wrapped with aluminium foil and homogenized. The medium was subsequently autoclaved at 121°C for 15minutes.

**Preparation of Potato Dextrose Agar (PDA)**

PDA was used for assaying fungi. It was prepared by dissolving 38grams of PDA powder in 1000ml of distilled water and was heated to dissolve with occasional shaking to avoid burning. The mouth of the conical flask was plugged with cotton wool and wrapped with aluminium foil and homogenized. The medium was then autoclaved at 121°C for 15minutes. After autoclaving and cooling to about 45°C, 2ml of streptomycin was added to the sterile Potato Dextrose Agar in a 250ml Erlenmeyer flask in order to inhibit the growth of bacteria on the plates while permitting only fungal growth.

**Preparation of MacConkey Agar**

It was prepared by dissolving 25.77grams of MacConkey Agar powder in 500ml of distilled water. The mouth of the conical flask was plugged with cotton wool and wrapped with aluminium foil and homogenized. The medium was then autoclaved at 121°C for 15minutes.
Preparation of De Man Rogosa and Sharpe (MRS) Agar

It was prepared by dissolving 33.55 grams of MRS powder in 500 ml of distilled water. The mouth of the conical flask was plugged with cotton wool and wrapped with aluminium foil and homogenized. The medium was the autoclaved at 121°C for 15 minutes.

Slopes or slants of PDA and NA were prepared by allowing the molten sterile agar to solidify at an angle of 45°C in sterile MacCartney bottles.

2.4 DETERMINATION OF PHYSIOCHEMICAL PARAMETERS

2.4.1 PHYSICAL OBSERVATION

The different brands of yoghurt drinks on arrival to the laboratory were visually observed for packaging conditions, colour and texture and these were recorded accordingly before microbiological investigations were carried out on them.

2.4.2 pH

This was determined using TDS pH meter. The pH meter was first switched on and the electrode was dipped into buffers of pH of 4 and 9 to confirm the accuracy of the meter and standardize it. The electrode was then withdrawn and the standby knob was switched off. The electrode was rinsed with distilled water and wiped dry using clean tissue paper and thereafter dipped into the yoghurt sample in question. The standby knob was then switched on to put the meter on. The reading was then taken where the pointer appeared to be stable. The electrode was withdrawn and then returns knob was depressed to place the pointer at 7. The electrode was then rinsed and wiped dry with clean tissue paper and this was repeated for the other brands of yoghurt.
2.4.3 TITRATABLE ACIDITY

The amounts of acid in the yoghurt drinks were determined by titrimetric method. The titratable acidity of all the different brands of yoghurt was determined by titration with 0.1N sodium hydroxide solution which was prepared by dissolving 2.0g of NaOH pellets in 500ml of distilled water. Each sample was diluted to ratio 1:10 with distilled water and 25ml of it was pipetted into clean conical flask. About 2-3 drops of phenolphthalein indicator was added to the diluted sample and titrated against 0.1N sodium hydroxide solution. A faint but permanent pink colour change marked the end point. Titratable acidity was expressed as the amount or volume of 0.1N sodium hydroxide that neutralized the sample, i.e. the volume of 0.1N NaOH titrated against 25ml of sample to raise its pH to 7.0.

2.4.4 MOISTURE CONTENT

A clean crucible was oven dried and weighed as (W₁), then about 10ml of the yoghurt was dispensed into it and both the crucible and the yoghurt sample were weighed and recorded as (W₂). The crucible and its content was then dried at 105°C in an oven for 24 hours after which it was removed and weighed again as (W₃) which gave a constant and final weight. This was done in duplicates and the average or mean was taken. The loss in weight represents the moisture content and the percentage was calculated as follows;

\[
\% \text{ Moisture content} = \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100.
\]
2.4.5 TOTAL SOLIDS

The total solids content of each yoghurt brand was also determined after oven drying at 105°C for 24 hours as described for moisture content above. The percentage was calculated with the formula below.

\[
\% \text{ Total solids} = \frac{\text{Final weight}}{} \times 100.
\]

2.4.6 ASH CONTENT

A clean oven dried crucible was weighed (\(W_1\)). 10ml of the yoghurt sample was added into the clean dried crucible and weighed as \(W_2\). The crucible and its contents was then transferred into the murtle furnace set at 600°C for about 6 hours, the colour change to ash showed that it was fully ashed. The crucible and its contents were removed from the furnace and placed inside desiccators to cool, after cooling it was then weighed as \(W_3\). The ash content of each of the samples was calculated as follows;

Weight of dried crucible = \(W_1\)

Weight of dried crucible + sample = \(W_2\)

Weight of dried crucible + sample after ashing = \(W_3\)

Weight of sample (\(W_S\)) = \((W_2 - W_1)\) g

Weight of Ash (\(W_A\)) = \((W_3 - W_1)\) g

\[
\% \text{ Ash} = \frac{W_3 - W_1}{W_2 - W_1} \times 100 \text{ i.e. } \frac{W_A}{W_S} \times 100
\]
2.5 ISOLATION OF MICROORGANISMS

Pour plate techniques were used for both bacterial and fungal isolation.

2.5.1 ISOLATION OF BACTERIA AND TOTAL BACTERIAL COUNTS

Ten (10) fold serial dilutions of the samples were made up to $10^{-5}$ as follows; 1ml of each yoghurt sample was drawn aseptically using a sterile needle and syringe and transferred into a test tube containing 9ml of sterile distilled water. After shaking, 1ml of the first diluted sample ($10^{-1}$) was aseptically withdrawn and transferred into another 9ml of sterile distilled water contained in a test tube and shaken again, this represents $10^{-2}$. The dilution was done up to $10^{-5}$. Subsequently 1ml each from dilutions $10^{-1}$, $10^{-3}$ and $10^{-5}$ was aseptically taken and plated on nutrient agar (NA) using the pour plate method. This was used for the total bacterial count.

Each of the plates was incubated at 37°C for 24 hours. Colonies that developed on the plate after incubation were observed and counted and records were made accordingly. Pure cultures were made by sub-culturing distinct colonies using the streak plate technique on nutrient agar until a clear distinct colony was obtained. The pure colony obtained was inoculated on a nutrient agar slant in a MacCartney bottle and incubated at 37°C for 24 - 48 hours and this was stored in the refrigerator as stock culture.

2.5.2 ISOLATION OF ENTERIC BACTERIA

After the serial dilution of the yoghurt drinks, 1ml each from dilutions $10^{-1}$, $10^{-3}$ and $10^{-5}$ was aseptically taken and plated on MacConkey agar using pour plate method. This was used for the enteric bacteria count.
Each of the plates was incubated at 37°C for 24 hours. Colonies that developed on the plate after incubation were observed and counted and records were made accordingly. Pure cultures were made by sub-culturing distinct colonies using the streak plate technique on nutrient agar until a clear distinct colony was obtained. The pure colony obtained was inoculated on a nutrient agar slant in a MacCartney bottle and incubated at 37°C for 24 - 48 hours and this was stored in the refrigerator as stock culture.

2.5.3 ISOLATION AND ENUMERATION OF LACTIC ACID BACTERIA

After the serial dilution of the yoghurt drinks, 1ml each from $10^{-1}$, $10^{-3}$ and $10^{-5}$ dilutions was aseptically taken and plated on De Man Rogosa and Sharpe (MRS) agar using pour plate method. This was used for the isolation and count of lactic acid bacteria.

Each of the plates was sealed with masking tape and incubated anaerobically using the GasPak technique for 48-72 hours (2-3 days) at 37°C. Colonies that developed on the plate after incubation were observed and counted and records were made accordingly. Pure cultures were made by sub-culturing distinct colonies using the streak plate technique on nutrient agar until a clear distinct colony was obtained. The pure colony obtained was inoculated on a nutrient agar slant in a MacCartney bottle and incubated at 37°C for 24 - 48 hours and this was stored in the refrigerator as stock culture.

2.5.4 ISOLATION OF FUNGI AND TOTAL FUNGAL COUNT

Following the serial dilution of the yoghurt, 1ml each from $10^{-1}$, $10^{-3}$ and $10^{-5}$ dilutions was aseptically taken and plated on Potato Dextrose Agar (PDA) using the pour plate method. This was used for the isolation of fungi and fungal count. The plates were incubated at 25°C for 3-5 days. Colonies that developed on the plate after incubation were observed and counted and
records were made accordingly. Pure cultures were made by sub-culturing distinct colonies using the streak plate technique on Potato Dextrose Agar until a clear distinct colony was obtained. The pure colonies obtained were picked with a sterile inoculating needle and inoculated into sterile PDA slant in McCartney bottle and incubated at 25°C for 3-5 days and this was stored in the refrigerator as stock culture.

**2.6 CHARACTERIZATION AND IDENTIFICATION OF BACTERIAL ISOLATES**

### 2.6.1 COLONIAL MORPHOLOGY

The bacterial colonies were first described and characterized by their morphological appearances (i.e. colony shape, edge or margin, pigmentation, elevation, colony surface, consistency and optical characteristics) on the plate. In addition to the colonial characterization, cellular morphologies and biochemical characteristics as described in the Laboratory manual of microbiology by Fawole and Oso (2007) were also used to characterize the bacteria. The isolates were subsequently identified using the Bergey’s manual of Determinative Bacteriology (Buchanan and Gibbons, 1974).

### 2.6.2 CELLULAR MORPHOLOGY

#### 2.6.2.1 Gram Staining

A drop of sterile distilled water was placed in the middle of a clean, grease free glass slide. A sterile wire loop was then used to pick a bacterial colony and a smear of the bacterial isolate was made on the slide. The smear was allowed to air dry and then heat fixed by passing the reverse side of the slide quickly through the flame three times. The slides were then flooded with crystal violet (primary stain) for 60 seconds, the stain was drained off and washed gently with distilled
water after which it was flooded with Lugol’s iodine (mordant) for 60 seconds and again drained off and washed gently with distilled water. It was then flooded with 95% alcohol for 15 seconds after which it was then rinsed with distilled water and finally counter stained with safranin for 30 seconds. The slide was then gently washed with distilled water and air dried. The slide was observed under oil immersion lens (× 100). Gram positive cells stained purple while Gram negative ones stained pink or red.

2.6.2.2 Motility test

The hanging drop method was used. Loop full of sterile distilled water was placed on a cover slip. A small portion of each bacterial isolate from 24 hours old culture was transferred to the drop of water on the cover slip using a sterile inoculating loop and a smooth suspension was made by thoroughly mixing it. Vaseline was applied around the edges of the cover slip to disallow air and it was carefully covered with a clean cavity glass slide. Cover-slip was pressed down to make an air tight seal. The cover slip was subsequently observed inverted upon the cavity slide under the ×40 objective lens. Motile bacterial cells were seen moving rapidly in the field (Fawole and Oso, 2007).

2.6.3 BIOCHEMICAL TESTS

All the bacterial isolates were subjected to a number of biochemical tests which are as follows.

2.6.3.1 Catalase test

Most aerobic microorganisms produce catalase enzyme that is capable of reacting with hydrogen peroxide (H₂O₂) to release oxygen. A few drops (i.e. 2 to 3 drops) of 3% hydrogen peroxide were placed in the middle of each clean slide and sterilized inoculating loop was used to pick bacterial
isolate from pure culture. This was used to make a smear on the slide containing hydrogen peroxide. The occurrence of effervescence caused by liberation of oxygen indicated production of catalase by the bacteria.

2.6.3.2 Citrate Utilization

The Simmon’s citrate agar slant in test tubes was used to carry out this test. Colony was picked with a sterilized inoculating loop by flaming and was used to streak the slants in the test tubes. The test tubes were incubated at 37°C for 72 hours and the colour change from green to blue was observed which indicated a positive result. No change indicated a negative result.

2.6.3.3 Coagulase test

Smears of various bacterial isolates were made on different slides and a drop of plasma was added. The slide was rocked for about 10 seconds and observed immediately for indications of agglutination or clumping. The clumping of organism in about 10 seconds indicated a positive result.

2.6.3.4 Triple Sugar Ion (TSI)

TSI agar was prepared as instructed by the manufacturer. Exactly 5ml of TSI powder was dispensed into each test tube and plugged with cotton wool, wrapped with aluminium foil and autoclaved at 121°C for 15 minutes. The test tubes were placed in a slanting position and allowed to solidify. Isolates were inoculated aseptically by stabbing the butt and streaking the slant. This process was repeated for all the slants with different colonies of bacteria. The tubes were incubated at 37°C for 24 hours. Yellow colouration of the agar indicated acid producing organisms while red colouration indicated alkali producing organisms.
2.6.3.5 Oxidase test

This test indicates the presence of cytochrome c oxidase that is able to reduce oxygen (O$_2$) and artificial electron acceptors (Prescott et al., 2008). A drop of 1% tetra methyl-p-phenylenediamine hydrogen chloride was dropped on a filter paper. Fresh culture of the isolate was then rubbed on the filter paper and observed. A possible result was indicated by a purple colour change within 10 seconds.

2.6.3.6 Indole Production

The test demonstrates the ability of some bacteria especially Gram negative to decompose tryptophan (an amino acid) to indole which is present in tryptone soya broth. Sterilized Tryptone soya broth (TSB) was inoculated with bacterial isolates in test tubes and incubated at 37$^\circ$C for 72 hours. After the incubation period, 2ml of chloroform was added to the broth cultures in each test tube and was shaken gently; thereafter 2ml of kovac’s reagent was added to each test tube and also shaken gently. The test tubes were returned to the test tube rack and were allowed to stand for about 20 minutes. A red colouration on the surface layer indicated indole production by the bacterial isolate which is a positive result (Fawole and Oso, 2007).

Methyl Red-Voges Proskauer (MR-VP) Test

Test tubes containing sterilized MR-VP broth were inoculated with the isolate in duplicate and incubated at 37$^\circ$C for 72hours. After incubation, the MR-test and VP-test were performed on each of the duplicate test tubes separately.
2.6.3.7 Methyl Red

To one of the test tubes, 5 drops of methyl red indicator was added and a change in colour was watched out for. A bright red colour on the surface indicated a positive result while a yellow or orange colour indicated a negative result.

2.6.3.8 Voges Proskauer

To the second test tube, 1ml of 5% α-naphthol solution was added followed by 1ml of potassium hydroxide (KOH) solution. The mixture was shaken and allowed to stand for some minutes and observed. A red colour within 5 minutes was indicative of a positive reaction.

2.6.3.9 Starch Hydrolysis

The ability of some bacteria to hydrolyze starch is detected by the presence of enzyme amylase. Soluble starch was added to already prepared nutrient agar in the ratio 2g soluble starch to 1 liter of nutrient agar and it was sterilized in the autoclave. The medium was thereafter poured into Petri dishes, inoculated and then incubated at 37ºC for 48hours. After incubation, the plates were flooded with Gram’s iodine solution and observed. A clear zone around a distinct colony indicated hydrolysis of starch (a positive result) while a blue-black colouration gave a negative result (Fawole and Oso, 2007).

2.6.3.10 Oxygen Relationship

Using a sterile inoculating loop, isolates were stabbed to a depth of about 6cm in sterile nutrient agar slants prepared in test tubes. The test tubes were then incubated at 37ºC for 24hours and observed. Aerobes grew at the surface while facultative anaerobes grew throughout the length of the stab.
2.6.3.11 Urease production

This is carried out to check the ability of bacterial isolates to hydrolyze urea (an organic nitrogen source) to ammonia and carbon dioxide by the production of an enzyme called urease. This distinguishes between certain members of the Enterobacteriaceae. Christein’s agar was inoculated with the bacterial isolates and incubated at 37°C for 7 days. It was observed daily for colour change. A change in colour from light orange to pink indicated a positive result.

2.6.3.12 Nitrate reduction

Nutrient broth was prepared according the manufacturer’s instruction by dispensing 1.3g of the powder into 100ml distilled water in an Erlenmeyer flask. The medium was heated over a Bunsen burner flame to homogenize it, and then 1ml of Sodium Nitrate (NaNO₃) solution was added to the broth after which it was distributed into McCartney bottles and autoclaved at 121°C for 15 minutes. The broths in the bottles were subsequently inoculated with the test organisms and incubated at 37°C for 72 hours. After 3-day incubation, few drops of 1% α-Naphtol was added to the culture and observed. A brownish ring on the top layer of the culture medium indicated a positive result.

2.6.3.13 Sugar Fermentation

The sugars used were lactose, glucose, sucrose, maltose, mannitol and fructose. These sugars are chiefly carbohydrate. Phenol red was used as an indicator; 10 ml of sterilized nutrient broth was dispensed into sterile test tubes and inoculated with the test organism. 0.5% (5g) of each sugar was weighed and poured into the test tubes, the test tubes were shaken gently to dissolve the sugars, and then about 5 drops of phenol indicator was introduced into the test tube and shaken to mix. Sterile Durham tubes were inserted in an inverted position into each of the test tubes, the
mouth of the test tubes were then plugged with cotton wool and incubated at 37°C for 7 days. The test tubes were examined after 24 hours for colour change, acid and gas production until the 7th day. On the 7th day, test tubes were then observed for acid and/or gas production in the Durham tubes (Fawole and Oso, 2007).

### 2.7 CHARACTERIZATION AND IDENTIFICATION OF FUNGAL ISOLATES

The fungal isolates were identified based on their colonial morphology (Gross identification) and cellular morphology by (Microscopic examination) using stained wet mount which showed the vegetative and reproductive structures of the fungal isolates.

#### 2.7.1 Colonial Morphology (Gross identification)

The parameters used in describing the colonial morphology were: colour of the hyphae, colour of the spores, size, shape, surface texture and elevation (Campbell and Stewart, 1980).

#### 2.7.2 Cellular Morphology (Microscopic examination)

Microscopic examination of stained wet mount was used in describing the cellular morphology. A drop of lactophenol cotton blue stain was placed on a clean slide. Two inoculating needles were held in both hands, one was used to remove a small piece of mycelium free of the medium from the agar. The mycelium was transferred onto the stain on the slide and teased apart with both needles. Cover slip was then used to cover it while avoiding bubbles in the process. The slide was examined under the X40 objective lens. Appropriate drawings and notes were recorded upon observation (Fawole and Oso, 2007).
CHAPTER THREE

RESULTS

The visual properties and packaging conditions of the eight (8) yoghurt drinks sampled in Ilorin metropolis as observed with the naked eye are presented in table 1 below.

Table 1: Physical characteristics and visual observation of the yoghurt samples

<table>
<thead>
<tr>
<th>S/N</th>
<th>Yoghurt drinks</th>
<th>Packaging</th>
<th>Colour</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Abide</td>
<td>Plastic container</td>
<td>Creamy white</td>
</tr>
<tr>
<td>2.</td>
<td>Hollandia (with strawberry)</td>
<td>Tetrapack sachet</td>
<td>Pink</td>
</tr>
<tr>
<td>3.</td>
<td>D-matrix</td>
<td>Plastic container</td>
<td>White and milky</td>
</tr>
<tr>
<td>4.</td>
<td>Fresh</td>
<td>Tetrapack sachet</td>
<td>Creamy</td>
</tr>
<tr>
<td>5.</td>
<td>Basako</td>
<td>Plastic container</td>
<td>Creamy white</td>
</tr>
<tr>
<td>6.</td>
<td>Ojoma</td>
<td>Plastic container</td>
<td>White and milky</td>
</tr>
<tr>
<td>7.</td>
<td>Savanna</td>
<td>Plastic container</td>
<td>Milky white</td>
</tr>
<tr>
<td>8.</td>
<td>Dudu</td>
<td>Plastic can</td>
<td>Creamy white</td>
</tr>
</tbody>
</table>
Physicochemical properties of the eight (8) yoghurt drinks from Ilorin metropolis

The physicochemical parameters investigated in the yoghurt drinks are pH, titratable acidity, moisture content, total solids and ash content. The results of the physicochemical tests are presented in table 2. The pH of the eight yoghurt drinks ranged between 3.51 and 4.25. Abide yoghurt had the lowest pH value of 3.51 and needed the highest volume of base (8.5ml) to neutralize it whereas Hollandia strawberry yoghurt recorded the highest pH value of 4.25 and needed the lowest volume of base (5.2ml) to raise its pH to 7.0. The moisture content of the yoghurt drinks ranged between 75.3% - 85.8% with ‘Savanna’ having the lowest while ‘Dudu’ had the highest. The total solids also varied from 14.2% - 24.7%, with ‘Dudu’ having the lowest and ‘Savanna’ the highest. The ash content ranged between 0.75% (for Hollandia) and 2.54% (for D-Matrix).
Table 2: Physicochemical characteristics of different brands of yoghurt in Ilorin

<table>
<thead>
<tr>
<th>S/N</th>
<th>Yoghurt brands</th>
<th>pH</th>
<th>Volume (ml) of base to raise pH to 7.0.</th>
<th>Moisture content</th>
<th>Total solids</th>
<th>Ash content</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Abide</td>
<td>3.51</td>
<td>8.5</td>
<td>81.7%</td>
<td>18.3%</td>
<td>1.90%</td>
</tr>
<tr>
<td>2</td>
<td>Hollandia</td>
<td>4.25</td>
<td>5.2</td>
<td>76.5%</td>
<td>23.5%</td>
<td>0.75%</td>
</tr>
<tr>
<td>3</td>
<td>D-matrix</td>
<td>3.62</td>
<td>8.0</td>
<td>85.4%</td>
<td>14.6%</td>
<td>2.54%</td>
</tr>
<tr>
<td>4</td>
<td>Fresh</td>
<td>3.56</td>
<td>8.2</td>
<td>78.9%</td>
<td>21.1%</td>
<td>0.86%</td>
</tr>
<tr>
<td>5</td>
<td>Basako</td>
<td>3.85</td>
<td>7.0</td>
<td>76.7%</td>
<td>23.3%</td>
<td>2.43%</td>
</tr>
<tr>
<td>6</td>
<td>Ojoma</td>
<td>4.08</td>
<td>6.8</td>
<td>79.2%</td>
<td>20.8%</td>
<td>1.41%</td>
</tr>
<tr>
<td>7</td>
<td>Savanna</td>
<td>3.58</td>
<td>7.5</td>
<td>75.3%</td>
<td>24.7%</td>
<td>1.36%</td>
</tr>
<tr>
<td>8</td>
<td>Dudu</td>
<td>4.16</td>
<td>5.5</td>
<td>85.8%</td>
<td>14.2%</td>
<td>0.79%</td>
</tr>
<tr>
<td></td>
<td><strong>MEAN</strong></td>
<td>3.83</td>
<td>7.1</td>
<td><strong>79.9%</strong></td>
<td><strong>20.1%</strong></td>
<td><strong>1.51%</strong></td>
</tr>
</tbody>
</table>
Results of the microbial enumeration of the eight yoghurt drinks in Ilorin

The average number of colonies obtained from the three plates was taken as the total microbial count comprising both bacteria (on NA) and fungi (on PDA). The counts are reported in table 3. The total bacterial count ranged between $0.23 \times 10^3$ CFU/ml (Basako yoghurt with the lowest count) and $4.10 \times 10^3$ CFU/ml (Abide yoghurt with the highest count). The total fungal count on the other hand showed that D-Matrix yoghurt had the lowest count ($0.01 \times 10^3$ CFU/ml) while Ojoma yoghurt had the highest ($1.24 \times 10^3$ CFU/ml). However, Fresh and Dudu yoghurt drinks did not contain any fungus. The result is presented in table 3.

Table 3: The microbial count of the eight (8) yoghurt drinks retailed in Ilorin metropolis

<table>
<thead>
<tr>
<th>S/N</th>
<th>Yoghurt brands</th>
<th>Bacterial count (CFU/ml) x $10^3$</th>
<th>Fungal count (CFU/ml) x $10^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Abide</td>
<td>4.10</td>
<td>1.02</td>
</tr>
<tr>
<td>2.</td>
<td>Hollandia</td>
<td>1.20</td>
<td>0.63</td>
</tr>
<tr>
<td>3.</td>
<td>D-matrix</td>
<td>2.35</td>
<td>0.01</td>
</tr>
<tr>
<td>4.</td>
<td>Fresh</td>
<td>0.45</td>
<td>Nil</td>
</tr>
<tr>
<td>5.</td>
<td>Basako</td>
<td>0.23</td>
<td>0.75</td>
</tr>
<tr>
<td>6.</td>
<td>Ojoma</td>
<td>2.92</td>
<td>1.24</td>
</tr>
<tr>
<td>7.</td>
<td>Savanna</td>
<td>2.60</td>
<td>1.18</td>
</tr>
<tr>
<td>8.</td>
<td>Dudu</td>
<td>0.38</td>
<td>Nil</td>
</tr>
<tr>
<td></td>
<td>MEAN</td>
<td>1.78</td>
<td>0.60</td>
</tr>
</tbody>
</table>
Result of Lactic acid bacteria (LAB) enumeration of the eight yoghurt drinks in Ilorin

The lactic acid bacteria (LAB) expected and isolated from the yoghurts using De Man Rogosa Sharpe (MRS) Agar were counted and reported below in table 4. The LAB counts of the eight yoghurt drinks were generally low compared to recommended standards. Hollandia yoghurt had the lowest LAB count with only about $0.56 \times 10^3$ CFU/ml while Savanna yoghurt had the highest LAB count containing $4.62 \times 10^3$ CFU/ml.

Table 4: The Lactic acid bacteria (LAB) count of eight (8) yoghurt drinks in Ilorin

<table>
<thead>
<tr>
<th>S/N</th>
<th>YOGHURT SAMPLES</th>
<th>LAB COUNT (CFU/ml) X 10³</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Abide</td>
<td>3.85</td>
</tr>
<tr>
<td>2.</td>
<td>Hollandia</td>
<td>0.56</td>
</tr>
<tr>
<td>3.</td>
<td>D-matrix</td>
<td>1.94</td>
</tr>
<tr>
<td>4.</td>
<td>Fresh</td>
<td>1.26</td>
</tr>
<tr>
<td>5.</td>
<td>Basako</td>
<td>3.71</td>
</tr>
<tr>
<td>6.</td>
<td>Ojoma</td>
<td>2.53</td>
</tr>
<tr>
<td>7.</td>
<td>Savanna</td>
<td>4.62</td>
</tr>
<tr>
<td>8.</td>
<td>Dudu</td>
<td>1.58</td>
</tr>
<tr>
<td></td>
<td>MEAN</td>
<td>2.51</td>
</tr>
</tbody>
</table>
Characterization and identification of bacterial isolates

The bacterial isolates were characterized and identified based on colonial and cellular morphological features as well as biochemical tests as presented in table 5 below. Table 5 shows the colonial morphology (macroscopic observation of colony on plates) and the cellular morphology (microscopic characteristics) of the bacteria isolated from the yoghurt samples.

Table 5: Colonial and cellular morphology of the bacterial isolates from eight (8) yoghurt

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Colony shape</th>
<th>Elevation</th>
<th>Edge</th>
<th>Optical characteristics</th>
<th>Consistency</th>
<th>Pigmentation</th>
<th>Colony surface</th>
<th>Gram stain</th>
<th>Cellular morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>Irregular</td>
<td>Flat</td>
<td>Undulate</td>
<td>Translucent</td>
<td>Butyrous</td>
<td>Pink</td>
<td>Smooth</td>
<td>_</td>
<td>Cocci</td>
</tr>
<tr>
<td>B2</td>
<td>Irregular</td>
<td>Flat</td>
<td>Lobate</td>
<td>Opaque</td>
<td>Butyrous</td>
<td>Creamy yellow</td>
<td>Wrinkled</td>
<td>_</td>
<td>Rods</td>
</tr>
<tr>
<td>B3</td>
<td>Circular</td>
<td>Raised</td>
<td>Entire</td>
<td>Opaque</td>
<td>Butyrous</td>
<td>Light yellow</td>
<td>Smooth</td>
<td>+</td>
<td>Cocci in clusters</td>
</tr>
<tr>
<td>B4</td>
<td>Irregular</td>
<td>Flat</td>
<td>Dentate</td>
<td>Opaque</td>
<td>Viscid</td>
<td>Creamy yellow</td>
<td>Wrinkled</td>
<td>+</td>
<td>Cocci</td>
</tr>
<tr>
<td>B5</td>
<td>Circular</td>
<td>Raised</td>
<td>Entire</td>
<td>Opaque</td>
<td>Butyrous</td>
<td>White</td>
<td>Smooth</td>
<td>+</td>
<td>Cocci in short chains</td>
</tr>
<tr>
<td>B6</td>
<td>Circular</td>
<td>Flat</td>
<td>Entire</td>
<td>Opaque</td>
<td>Butyrous</td>
<td>Creamy white</td>
<td>Dry</td>
<td>+</td>
<td>Single rods with round ends</td>
</tr>
</tbody>
</table>
The bacteria were characterized based on their reactions to the various biochemical tests and subsequently identified using Bergey’s Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974). The reactions of the bacterial isolates to the various biochemical tests performed on them are recorded in table 6 below and the probable bacteria are subsequently reported.

**Table 6: Biochemical characterization and identification of bacteria**

| Isolates | Catalase | Coagulase | Indole | Triple sugar ion | Methyl red | Voges proskauer | Nitrate reduction | Citrate utilization | Urease production | Starch hydrolysis | Motility | Oxidase | Oxygen | Lactose | Maltose | Mannitol | Sucrose | Glucose | Fructose | Probable bacteria                      |
|----------|---------|-----------|--------|------------------|------------|-----------------|------------------|-------------------|-------------------|------------------|----------|---------|--------|---------|---------|---------|---------|---------|---------|---------|-----------------------------|
| B1       | +       | -         | AL     | +                | -          | +               | +                | +                 | -                 | +                | +        | +       | +      | A       | AG      | A       | AG      | A       |    A    | F        | *Serratia marcescens*        |
| B2       | -       | -         | A      | +                | -          | +               | +                | -                 | +                 | FA               | A        | AG      | AG     | AG      | AG      | AG      | AG      | AG      | AG      | AG      | *Proteus mirabilis*          |
| B3       | +       | +         | A      | +                | -          | +               | -                | +                 | -                 | +                | +        | AE      | A      | A       | A       | A       | A       | A       | A       | A       | *Staphylococcus aureus*       |
| B4       | +       | -         | A      | +                | -          | +               | +                | +                 | +                 | -                | AE       | A       | A      | A       | A       | A       | A       | A       | A       | A       | *Micrococcus luteus*         |
| B5       | -       | -         | A      | -                | -          | +               | +                | -                 | +                 | -                | FA       | A       | A      | A       | A       | A       | A       | A       | A       | A       | *Streptococcus thermophilus*  |
| B6       | -       | -         | A      | -                | -          | +               | +                | -                 | +                 | -                | FA       | A       | A      | A       | A       | A       | A       | A       | A       | A       | *Lactobacillus bulgaricus*    |

**KEY:** + = positive; - = negative; AE = aerobic; FA = facultative anaerobe; A = acid production; AL = alkali; AG = acid and gas.
Distribution and occurrence of bacteria in the yoghurt brands from Ilorin metropolis

Six (6) different bacterial species were isolated from the eight yoghurt samples namely *Serratia marcescens, Proteus mirabilis, Staphylococcus aureus, Micrococcus luteus, Streptococcus thermophilus and Lactobacillus bulgaricus*. The first four bacteria were contaminants in the yoghurts and therefore undesirable while the last two are desirable microorganisms as they constitute the starter cultures used in the fermentation of milk to produce yoghurt. *Staphylococcus aureus* was found in 50% of the eight yoghurt drinks analyzed. Though in very low quantity, *Lactobacillus bulgaricus* was found in almost all the yoghurt drinks with the exception of Hollandia while *Streptococcus thermophilus* was isolated only from four (4) of the yoghurt drinks namely Abide, Hollandia, Basako and Dudu but absent in others. Only three (3) yoghurt brands (Abide, Basako and Savanna) had the expected combination of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*.

The bacterial isolates are reported in table 7 according to their occurrence and distribution in the various yoghurt samples.
Table 7: Distribution of bacterial isolates from yoghurt brands in Ilorin metropolis.

<table>
<thead>
<tr>
<th>YOGHURT BRANDS</th>
<th>Serratia marcescens</th>
<th>Proteus mirabilis</th>
<th>Staphylococcus aureus</th>
<th>Micrococcus luteus</th>
<th>Streptococcus thermophilus</th>
<th>Lactobacillus bulgaricus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abide</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hollandia</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>D-matrix</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Fresh</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Basako</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ojoma</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Savanna</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Dudu</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

**KEY:** + = present

- = absent
Distribution and occurrence of fungi in the yoghurt brands from Ilorin metropolis

Five (5) different fungal species were isolated from the eight yoghurt drinks analyzed. The isolated fungi were *Rhizopus stolonifer*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Mucor racemosus* and *Mucor spp.*

*Rhizopus stolonifer* and *Mucor racemosus* were found in half (4) of the eight yoghurt drinks analyzed whereas the others were distributed in one or two samples. The five fungi species isolated from the yoghurt samples are reported in table 8 below according to their distribution.

Table 8: Distribution of fungal isolates from different yoghurt brands in Ilorin metropolis.

<table>
<thead>
<tr>
<th>Samples</th>
<th><em>Rhizopus stolonifer</em></th>
<th><em>Aspergillus fumigatus</em></th>
<th><em>Aspergillus flavus</em></th>
<th><em>Mucor racemosus</em></th>
<th><em>Mucor spp.</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Abide</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Hollandia</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>D-matrix</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Fresh</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
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**KEY:** + = present  
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Identification and brief description of fungal isolates

Colonial characteristics and microscopic examination of stained wet mount were used in identifying the fungi isolated from the different yoghurt samples. Cellular morphology as observed under the microscope was recorded and appropriate drawings were made.

**Isolate F1:** The fungus grew into maturity on PDA within 5 days of incubation at room temperature. Colonies produced black fluffy colouration with powdery appearance. Microscopic observation after staining with Lactophenol-cotton blue revealed non-septate hyphae with short, stout and stiff sporangiophores, each bearing a sporangium enclosing black sporangiospores. Horizontal hyphae called stolons were also seen and rhizoids (root-like) hyphae attached beneath. The fungus was subsequently identified as *Rhizopus stolonifer*.

**Isolate F2:** The fungus also grew on PDA within 3-5 days, producing a pure white mycelium which appeared cottony. Microscopic examination revealed septate hyphae without sporangium. The mycelia also appeared bushy. The fungus was subsequently identified as *Aspergillus fumigatus*.

**Isolate F3:** The fungus produced a pale green mycelium on PDA within 3-5 days of incubation. The mycelium appeared green at the centre, lining at the exterior with white fluffy. Under the microscope, septate hyphae bearing long conidia were seen. Each conidium produced numerous conidiospores were being dispersed. The fungus was subsequently identified as *Aspergillus flavus*. 
**Isolate F4:** The fungal mycelium appeared black on a PDA. Microscopic examination revealed long, delicate hyphae without cross-walls and rhizoids. The long non-septate sporangiophores bore sporangia some of which were ruptured, releasing the sporangiophores. This fungus was subsequently identified as *Mucor recemosus*.

**Isolate F5:** A grayish white mycelium that grew very rapidly on PDA, growing like a mass of cotton up to the lid of the Petri dish and spreading over the whole plate within 3 days. Its microscopic examination revealed long and delicate non-septate hyphae with sporangium without sporangiospores. Spores however were seen around the mycelia. The mycelia lacked horizontal hyphae and rhizoid. This fungus was subsequently identified as *Mucor spp.*
CHAPTER FOUR

DISCUSSION

The results of the physicochemical screening of the selected yoghurt drinks in Ilorin metropolis agreed with the results obtained in past work by some researchers in other parts of Nigeria. The pH of the eight (8) yoghurt drinks in this study ranged from 3.51 - 4.25 (Mean of 3.83) which is similar to the range of 3.69 - 4.09 obtained by Ifeanyi, et al (2013) who studied yoghurt quality in Onitsha, Southeastern Nigeria and equally in agreement with the range of 3.57 - 4.12 obtained by Bamise and Bamise (2007) who quantified the acidic contents of commercial yoghurt drinks in Nigeria. However the mean pH was below and significantly different from the mean pH of 4.28 obtained by Rodrigues, et al. (2010) who studied microbiological quality of yoghurts in Brazil. Although the pH is not an official parameter to verify the quality of yoghurt, it can be measured in order to allow additional information, Rodrigues, et al. (2010). In general, two methods used to quantify the acid content of a drink are the pH and titratable acidity (TA). The pH is a measure of the hydrogen ion concentration (H+) while the TA is the total number of acid molecules and determines the actual H+ availability (Boulton, 1980).

The moisture content varied from 75.3 to 85.8% with a mean of 79.9% which as expected is lower than the moisture content of raw milk as a result of fermentation. The total solids content of the yoghurts ranged between 14.2 to 24.7% (mean of 20.1%) which is significantly different from the findings of Muhammad et al. (2005) who reported the highest range of total solids in yoghurt to be 17.1%. The result is also slightly different from that obtained by Tamime and Robinson (2007) who reported that many commercial yoghurt products have milk solids contents of 14-15%. The ash content of the yoghurt samples also varied from 0.75 to 2.54%.
The labels on the yoghurt blends provided little information about the products which included only production date, expiry date, batch number and NAFDAC Registration number but excluded the nutritional composition. Moreover, the names and the amounts of the starter cultures used for the production which could help consumers to determine whether or not probiotic health benefits can be derived from their consumption were not provided on the labels.

The study of the microbiota of the selected yoghurt drinks in Ilorin Metropolis showed low levels of microbial counts for both contaminants and the desired starter cultures. The microbial analysis revealed a total of six (6) bacterial species and five (5) fungal species. The total bacterial count ranged from $0.23 \times 10^3$ CFU/ml to $4.10 \times 10^3$ CFU/ml. The total fungal count on the other hand varied between $0.01 \times 10^3$ CFU/ml and $1.24 \times 10^3$ CFU/ml.

The isolation of fungi such as Aspergillus, Mucor and Rhizopus species agreed with Oyeleke (2009) that moulds are the primary contaminants of yoghurt produced in Nigeria. Serratia marcesens, Proteus mirabilis, Staphylococcus aureus and Micrococcus luteus were bacterial contaminants in the yoghurts. No coliform was isolated from any of the yoghurt samples. Yoghurt is not expected to be sterile (free of microorganisms) as the heat treatment of the milk used for production only kills pathogenic microorganisms and substantially reduces the level of spoilage microorganisms. The presence of these contaminants therefore might be caused by inadequate heat treatment (Pasteurization) of milk and poor hygienic standards of processing and packaging that led to recontamination of the product. In addition, the microorganisms could have been introduced into the products from the skin microflora (e.g. S. aureus and Micrococcus) of personnel employed in the production or from the non-sterile production environment. The detection of fungi and other bacteria probably indicated post-production contamination. Furthermore, the detection of these contaminating microorganisms could also possibly indicate
post-production contamination as a result of storage under inappropriate conditions (above 10°C) during sales in the market environment. Post-production contamination was not impossible, considering the non-sterile environment in which production and sales were carried out. Although the levels of these contaminants were considerably low and safe at the time of analysis, if stored further for a longer period of time under inappropriate conditions, the contaminants would grow, multiply and attain a high level which could pose a health danger to the consumers. Attention of the stake holders including manufacturers and retailers is therefore needed to reduce postproduction contamination.

With respect to lactic acid bacteria (LAB) enumeration, all the eight (8) samples showed microbiological parameters that were not in conformity with official standards. The amounts of starter cultures were far lower than $10^7$ CFU/ml standard as reported by Rybka and Kailasapathy, (1995). The lactic acid bacteria (LAB) which constitute the starter cultures of the yoghurts had low counts varying between $0.56 \times 10^3$ CFU/ml and $4.62 \times 10^3$ CFU/ml, which were all far below the recommended $10^7$ CFU/ml needed to function as probiotics and exert some health benefits on the consumers. Since LAB enumeration indicates the levels of added starter culture and its development during the storage and shelf-life, it suggests that small amounts of the starter cultures were inoculated in all the eight samples of yoghurt analyzed. Low levels of LAB in fermented products can occur due to inappropriate conditions of storage leading to uncontrolled development of the starter cultures (Tamine, 2002; Tamine and Robinson, 2007), with consequent acid production. When yoghurt is kept in inappropriate storage conditions, the LAB from starter cultures tend to increase their development, provoking high acidity and consequently killing themselves (Tamine and Robinson, 2007). These findings are similar to the one obtained by Ifeanyi et al. (2013).
Only two LAB which comprise the starter cultures were isolated from all the yoghurt samples and they are *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. These starter cultures were not in the right proportion and only three (3) of the yoghurt drinks contained both organisms; others contained only one of the two in minute quantity. This unequal proportion of starter cultures was against the report of Tamime and Robinson (1999) that successful preparation of yoghurt depends upon the proper symbiotic relationship between the two organisms at equal proportion. However the isolation of the two organisms: *Streptococcus* and *Lactobacillus* spp. agreed with the claims that they are the most commonly employed starter cultures in the fermentation of milk into yoghurt (Steinkraus, 1997; Tamime and Robinson, 1999).
CONCLUSION

Based on the results of this study, it can be concluded that the locally produced yoghurts retailed in Ilorin metropolis presented inadequate microbiological quality and standards. Although the contamination loads were low, the yoghurts could constitute public health hazards if stored under inappropriate conditions for a longer period of time. Power supply is generally poor and epileptic in Nigeria and Ilorin city is not an exception from this challenge. The growth of the microbial contaminants was therefore encouraged due to lack of stable power supply which is needed to keep the yoghurts refrigerated always in order to maintain their quality and inhibit the growth of spoilage microorganisms. Furthermore, it can as well be ascertained that many brands of locally produced yoghurt being commercialized in Ilorin cannot confer any health benefits to the consumers considering the low levels of the starter cultures (i.e. Lactic acid bacteria). In other words, as far as probiotics and the associated health benefits are concerned, the locally produced yoghurts are nowhere to be found. This deficiency suggests inoculation of starter cultures in small amounts and it is an indication that the manufacturers are less concerned about the health benefits that could be derived from the consumption of yoghurt. Unlike in developed countries, local manufacturers in Ilorin specifically and in Nigeria as a whole are not motivated by the desire to promote public health and general well being of the populace through the production of yoghurts with adequate microbiological quality, rather they are driven by the selfish desire to only make huge profits.
RECOMMENDATIONS

In order to ensure the availability of yoghurts with good microbiological quality for consumers in Ilorin as a city and in Nigeria as a whole, the following measures are my recommendations:

- Local yoghurt manufacturers should try as much as possible to always ensure aseptic conditions in their production environment in order to prevent the contamination of their products during processing.

- The manufacturing companies should also ensure that their production personnel always maintain good personal hygiene so as to reduce the risk of contaminating the yoghurts with members of the normal microbiota of their skin, mouth, nose, etc. Nose masks should be worn always in the production room.

- Adequate pasteurization of raw milk must be ensured in order to eliminate all the pathogenic microorganisms and reduce to a substantial level other contaminating or spoilage microorganisms in the milk before it is used for yoghurt production.

- The manufacturers should be conscious of the fact that some therapeutic health benefits can be derived from yoghurt if produced properly and as such they should always try as much as possible to inoculate adequate amounts of starter cultures needed to attain about 10 million cells into the pasteurized milk for fermentation.

- The retailers or vendors should avoid storage of yoghurt on the shelves at room temperature, but rather keep them refrigerated always. To overcome the challenge of epileptic power supply, retailers should order only a small quantity of packaged yoghurts which they can sell out to the consumers within a short period of time.
Manufacturers should have adequate refrigerating facilities needed for the storage of their finished products in the factory prior to distribution rather than storing on the shelves and floors which could cause deterioration in the quality of their products.

In the transportation of yoghurt from factory to the market, manufacturers should endeavor to always transport their products in refrigerated vehicles in order to maintain the appropriate storage temperature up to the point of distribution. This would ensure a steady maintenance of their microbiological qualities.
REFERENCES


