QUALITY EVALUATION OF FERMENTED COW MILK (*NUNU)* CONSUMED IN SOME METROPOLISES IN SOUTHERN NIGERIA.

BY

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CERTIFICATION

This is to certify that this work: **Quality evaluation of fermented cow milk (*Nunu)* consumed in some metropolises in southern Nigeria** was done by EZENWEANI, EMMANUEL CHIDI of registration number 20164997098 in the Department of Microbiology, School of Biological Science, in partial fulfilment of the requirements for the award of Master of Science (M.Sc.) degree in Food/Industrial Microbiology.

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DEDICATION

This work is dedicated to my supervisor Prof. Wesley Braide for his scrutiny, support and guidance.

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ABSTRACT

The study was carried out to evaluate the quality of *Nunu* (fermented raw milk) taken at different sampling locations from Owerri, Obinze, Elele, Asaba and Enugu. The mean total bacterial count from these five locations were $9.55\log_{10}$cfu/ml, $9.67\log_{10}$cfu/ml, $9.58\log_{10}$cfu/ml, $9.57\log_{10}$cfu/ml, and $9.542\log_{10}$cfu/ml respectively, which showed no significant difference ($p>0.05$) between the different locations. The mean values were significantly higher ($p<0.05$) than the recommended level of $6.03\log_{10}$cfu/ml by East Africa Community Standards. The mean total *Campylobacter* count also showed no significant difference ($p>0.05$) between the different locations, with least mean value $3.49\log_{10}$cfu/ml from Owerri and Enugu. The mean total *Salmonella* count also showed no significant difference ($p>0.05$) with mean least value of $1.28\log_{10}$cfu/ml, and highest of $1.8695\log_{10}$fu/ml, from Asaba and Enugu locations respectively. There was significant difference ($p<0.05$) in *staphylococcal* count ($\log_{10}$cfu/ml) between the different locations, with samples from Elele recorded the highest mean value. The mean total coliform count showed no significant difference ($p>0.05$) between the different locations, with means being above standard. The isolated bacteria included *Staphylococcu*s sp, *Salmonella* spp, *Bacillus* spp, *Klebsiella* spp, *Campylobacter* spp, *Enterobacter* spp, *Enterococcus* spp, *Escherichia coli*, *Micrococcus* sp and *Shigella* spp. The biochemical test used to determine the milk quality such as Methylene blue and Resazurin test suggested high microbial contamination of the *Nunu* samples. Other biochemical tests such as alcohol test, Titrable acidity and Alkaline phosphatase activity were recorded high and was significantly ($p<0.05$) higher than the standard by Thai Agricultural Standard. The Temperature of the samples indicated good condition for bacterial growth as it ranged from 27.47^0C to 27.79^0C. The pH indicated an acidic condition (4.69 – 5.23) which must have been caused by lactic acid producing bacteria. This study revealed that *Nunu* samples analysed contained heavy metals such as cadmium, lead and zinc but within permissible level. The nutritional composition showed that the values for protein, fat, and carbohydrate were within permissible level. This study concluded that the quality of *Nunu* produced and marketed in these five cities in southern Nigeria was poor, and consumers may be at risk of contracting milk-borne infections from continuous consumption of *Nunu* from these locations as at the time of this study. It is recommended that public health officers and all stakeholders in the food and health industry should play their roles in order to ensure safe quality *Nunu* production.

Keywords: Quality, Contamination, *Nunu*, Heavy metals, Proximate composition.

CHAPTER ONE

1.0 INTRODUCTION

Milk is an important source of nutrients to human and animals. Milk has been man's best food from birth. Milk has a complex biochemical composition and with its high water activity and nutritional value, milk therefore serves as an excellent medium for growth and multiplication of many kinds of microorganisms when suitable conditions are maintained (Parekh & Subhash, 2008). Milk consumed by humans must be free from any pathogenic organism and must be of high quality (Bertu *et al.*, 2010).

According to Aliyu *et al*., (2019), milk can be effective in promoting muscle growth. *Nunu* as one of the fermented milk product in Nigeria is skimmed (defatted) milk. The implementation of 'School Milk Programmes' all over the world is an evidence to the realisation of nutritional attributes of milk (Aliyu *et al*., 2019). The major chemical constituents of milk include: water, fats, proteins, carbohydrates, minerals, organic acids, enzymes, and vitamins (Tamime, 2009). Milk composition is dependent on the producing species and the composition and properties of fresh cow's milk show considerable variability (Tamime, 2009). It was stated that the main factors responsible for such variability include; genetic factors (e.g bleed and individual), stage of lactation, health status of the cow and environmental factors (e.g feed climate or method of milking) (Walker *et al*., 2004; Tamime, 2009).

Nunu is one of the indigenous milk products in West Africa countries including Nigeria. It is very popular in the Northern part of Nigeria and every Hausa/Fulani communities around the country. *Nunu* is the Fulani word for fermented cow milk sold by the Fulani women. The poor handling of *Nunu* during processing exposes it to microbial contamination and it is highly susceptible to microbial spoilage (Otoikhian, 2012). Milk, if not properly handled can be a source of transfer of some pathogenic organisms from animals to humans; such as

Mycobacterium bovis and *Mycobacterium tuberculosis*, which causes animal and human Tuberculosis. Some microorganisms although not pathogenic, can induce off-flavour, curdling and rancidity of milk (Otoikhian, 2012).

1.1 Statement of Problem

Raw milk is an important vehicle for the transmission of milk-borne pathogens to humans, as it can be easily contaminated during milking and handling (Addo *et al*., 2011). In Nigeria a major part of the local milk production is done mainly by the Fulani who control more than 90% of the cattle population (Okeke *et al.,* 2016) and the milk produced is from indigenous cattle breeds which are kept primarily by the pastoralist tribesmen. The cattle are rarely given standard feeds, and the kinds of feed intake later reflect in the composition of their milk. To my best of knowledge, studies on the physico-chemical composition and proximate composition of *nunu* in southern Nigeria has not been reported. Therefore, due to the practice of open grazing method in keeping the cattle, and inadequate handling and processing of milk, there is need to assess the quality and safety of *nunu* produced and sold by Fulani women in southern part of Nigeria.

1.2 Aim of Study

To evaluate the quality of fermented Cow milk (*Nunu*) consumed in some metropolises in southern part of Nigeria.

1.3 Objectives of study

i. To determine total bacterial load of *Nunu* samples

ii. To determine total *Salmonella* load present in *Nunu* samples

iii. To determine total Coliform Count of the *Nunu* samples

iv. To determine total *Campylobacter* count of the *Nunu* samples

v. To determine total Staphylococcal count of the *Nunu* samples

vi. To determine the microbiological quality of the *Nunu* samples using chemical analytical methods such as Methylene blue test, Resazurin test, Acidity test, Alcohol test, and Alkaline phosphatase activity.

vii. To determine the physical conditions such pH and temperature of the *Nunu* samples.

viii. To determine the concentration of heavy metals such as lead, cadmium and zinc in the *Nunu* samples.

ix. To determine the proximate components of the *Nunu* samples.

1.4 Justification of Study

The information on the quality of *Nunu* consumed in Southern Nigeria is scarce, and only the level of microbial contamination of the product can be found from the literature available. Therefore, this study will bridge the gap on the quality information of *Nunu* consumed in Southern Nigeria.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Definition of Milk

Milk is a yellowish-white non-transparent liquid that is secreted by the mammary glands of all mammals. It contains all nutrients required by the neonate and has long been recognised as nature's ultimate food; it is also rich source of protective agents, enzymes and growth factors (Tamime, 2009)

2.2 Milk Composition

Milk is a colloidal suspension; containing emulsified globules of fat, a heterogeneous family of major and minor proteins, the carbohydrate lactose, minerals, vitamins and enzymes (Tamime, 2009).

Tamime, (2009) showed that milk composition is dependent on the producing species and the composition and properties of fresh cow's milk show considerable variability. It was stated that the main factors responsible for such variability include; genetic factors (e.g bleed and individual), stage of lactation, health status of the cow and environmental factors (e.g feed, climate or method of milking) (Walker *et al*., 2004; Tamime, 2009).

Table 2.1 Approximate composition (g/100g) of milk from cow species

Parameters	Composition (g/100g)
Total solids	12.7
Fat	3.7
Protein	3.4
Lactose	4.8
Ash	0.7

Source: Tamime, 2009

Table 2.2 Composition (g/100g) of cow's milk from various breeds

BREED	FAT	PROTEIN	LACTOSE	ASH	TOTAL SOLIDS
Ayrshinre	4.0	3.3	4.6	0.7	12.7
Brown Swiss	3.8	3.2	4.8	0.7	12.7
Guernsey	4.6	3.5	4.8	0.8	13.7
Holstein	3.6	3.0	4.6	0.7	11.9
Jersey	5.0	3.7	4.7	0.8	14.2

Source: Tamime, 2009

2.3 Microflora of Raw Milk

The types of organisms present in raw milk are influenced by temperatures and time of storage as well as methods of handling during and after milking (Varnam & Sutherland, 2001). Examples of microflora of raw milk include; *Pseudomonas* spp, *Bacillus* spp, *Esherichia coli, Streptococcus* spp, *Lactobacillus* spp, *Staphylococcu aureus, Campylobacter jejuni* etc. Some of the microfloras of raw milk are spoilage organisms and pathogenic organisms.

2.3.1 Spoilage Organisms

2.3.1.1 Gram negative psychrotrophic bacteria

The growth of psychotropic bacteria is of major concern when raw milk is kept at low temperature. During growth of these bacteria, heat-stable enzymes such as proteases and lipases are formed and cause protein and lipid degradation (Muir, 1996; Tamime, 2009).

2.3.1.1.1. *Pseudomonas* spp

They are motile, gram negative rods, with the ability to grow at temperatures just above freezing, despite their optimum growth temperature being between 25 and 30^0C (Tamime, 2009). They are most important because of their ability to produce heat-stable enzymes (particularly proteases and lipases) during growth under refrigerated storage (Donkor *et al*., 2007). Only 1 colony count unit of *Pseudomonas* spp as initial number present in raw milk is enough to cause spoilage within 5 days at 3-5^0C due to its rapid generation time of 5.5-10.5hrs (Donkor *et al*., 2007)

2.3.1.1.2 *Enterobacteriaceae*

They are small, gram negative rods. They account for 5-33% of psychrotrophic microflora present in raw milk (Bukuku, 2013). Coliforms belonging to this group are able to ferment lactose with production of acid and gas at 32^0C within 48hrs. Other psychrotrophic bacteria commonly found in raw milk include flavobacterium (Bukuku, 2013).

2.3.1.2 Gram positive bacteria

2.3.1.2.1 *Bacillus* spp:

They are gram positive, motile, sporeformers, rod-shaped organisms. The most dominant *Bacillus* spp commonly isolated from milk are *B. Lichenformis, B.cereus, B. Subtilis, and B. Megaterium* (Al-Tahiri, 2005). *Bacillus cereus* is a common contaminant of raw milks, being present in over 80% of raw milk samples (Vasavada & Cousin, 1993; Al-Tahiri, 2005).

2.3.1.2.2. *Clostridium* spp

They are present in raw milk at very low level that only enrichment and most probable number (MPN) are used for quantification (Al-Tahiri, 2005).

2.3.1.2.3. Lactic acid bacteria:

Spoilage of raw milk resulting from growth of acid-producing fermentative lactic acid bacteria occurs when storage temperatures are sufficiently high for these microorganisms to outgrow psychrotrophic bacteria or when product composition is inhibitory to gram negative aerobic organisms (Al-Tahiri, 2005). Species of *Streptococcus, Enterococcus, Lactobacillus, Leuconostoc, Lactococcus, and Pediococcus* are involved. *Lactococcus lactis subsp. Lactic* is the main species responsible for the spoilage of raw milk at 10-37^0C, being able to produce

acid (about 0.25%, mostly lactic acid but also small amounts of acetic acid and propanoic acid) to cause milk to sour (Al-Tahiri, 2005; Bukuku, 2013).

These microorganisms are a minor population of the micro-flora in raw milk, but their numbers may be proportionally higher in pasteurised milk because of their resistance to pasteurisation (Al-Tahiri, 2005).

2.3.2 Pathogenic Organisms

Numerous milk-borne pathogens have been isolated from raw milk and outbreaks of illness caused by *Campylobacter jejuni*, Shiga toxin producing *Esherichia coli, Staphylococcus aureus, Listeria monocytogenes, Salmonella spp and Yersimia enterocolitica* have been reported following consumption of milk (Steele *et al*., 1997; Baek *et al*., 2000).

The pathogenic organisms are of two types: those involved in bovine mastitis and those that externally contaminate milk. The bacteria causing mastitis include *S. aureus, Streptococcus agalactiae, Str. Dysgalactice, Str. uberis, Listeria* spp *and E. coli (*Hillerton & Berry, 2005). The *Staphylococcus aureus* are of public health concern due to its ability to produce heat-stable enterotoxins that can cause food poisoning. *Str. agalactiae* causes bacteraemia and meningitis, which are fatal to infected infants (Chambers, 2002).

The pathogenic organisms that externally contaminate milk are *Salmonellae* and thermoduric *Campylobacter* strains. They are from sources external to the udder (Chambers, 2002). *Salmonellae* are major public health concern especially now that there is emergence of multiple antibiotic-resistant strains of *Salmonella typhimurium DT104* (El-Gazzar & Marth, 1992; Tamime, 2009).

2.4 Sources of Microbial Contamination in Milk

Milk as a nutritious food is prone to microbial contamination and many milk-borne epidemics of human are spread through contaminated milk (Nonga *et al.*, 2015). This is true especially in developing countries where milk and milk products production and handling takes place under unhygienic environment and poor production practices.

Milk is sterile when it is in the udder of a healthy animal but becomes contaminated with bacteria mainly during and/or after milking (Makerere University, 2011; Karimuribo *et al*., 2015). Milk from subclinical mastitic cows usually contains aetiological agents but milk from non-mastitic cows is often contaminated from extraneous dirt or poor quality water (Kivaria *et al.*, 2006).

2.4.1 The Cow

Milk from a healthy cow has a low bacterial count (Mboya, 2016). A cow sheds microorganisms into milk when it is sick (Mboya, 2016). Examples of microorganisms include *Streptococcus agalactiae* and *Staphylococcus aureus* which are found in the infected udder of mastitic cows (Mboya, 2016). Milk from a sick cow should not be mixed with the one from healthy cows and sick cows should be milked last to avoid contamination of the clean milk.

2.4.2 The Milker

Milkers can transmit contagious diseases such as Tuberculosis and Brucellosis. They can transmit these diseases through their hands, hair, clothing and aerosols from sneezing or coughing (Karimuribo *et al.*, 2015; Swai & Schoonman, 2011; Mboya, 2016).

2.4.3 The Milking Environment

This includes the skin of the animal, feeds, containers and water facilities. It is important to keep the environment around the dairy animals as clean as possible. Also, bedding materials must be kept dry and clean at all times (Maunsell & Donovan, 2008)

2.4.4 Milk containers

When containers used to store milk after milking are not thoroughly cleaned they harbour pathogens and spoilage microorganisms which will contaminate the milk (Kanyeka, 2014).

2.5 Milk-borne Infections and Pathogenic Microorganisms

Various bacteria may have access to milk and milk products from different sources and cause different types of milk-borne illnesses. Sometimes milk and milk products may carry microorganisms or their toxic metabolites (poisons/toxins). Some of these microorganisms are pathogenic and cause illness to humans while others cause spoilage in milk rendering it unsuitable (unsafe) for human consumption (Kivaria *et al.,* 2006; Parekh & Subhash, 2008; Bukuku, 2013). Many milk-borne epidemics of human diseases are spread through consumption of contaminated milk (Parekh & Subhash, 2008). Few examples of the known milk-borne diseases are Bovine tuberculosis, Brucellosis, Anthrax, Listeriosis, Salmonellosis, Leptospirosis, Q-fever, Campylobacteriosis and *E. coli* O157:H7 as an emerged new milk-borne bacterial pathogen reported recently with a very serious health effects (Sivapalasingams *et al*., 2004). These are zoonotic diseases which are transmitted to consumers and pose a risk to public health. To protect consumers and public health against these milk-borne infections it requires proper hygienic milking and milk handling procedures.

Common bacteria reported to be isolated from milk include *Staphylococcus* spp., *Listeria* spp., *Salmonella* spp., *E. coli, Campylobacter* spp., *Mycobacterium* spp., *Brucella* spp., *Coxiella*

burnetii, *Yersinia* spp., *Pseudomonas aeroginosa* and *Corynebacterium ulcerans.* Others are *Proteus* spp., *Leptospira* spp., *Clostridium* spp., *Streptococcus* spp, *Klebsiella* spp., *Enterobacter* spp. and *Bacillus* spp (Shirima *et al.*, 2003; Sivapalasingams *et al.*, 2004; Al-Tahiri, 2005; Donkor *et al*., 2007; Parekh & Subhash, 2008). All these are pathogenic bacteria that pose serious threat to human health and contribute up to 90% of all dairy related diseases (De Buyser *et al*., 2001; Sivapalasingams *et al.*, 2004; Donkor *et al*., 2007).

Therefore, proper milking, cleaning and sanitizing procedures of equipments and environments are essential tool to ensure quality of milk. Pasteurization of milk has shown to reduce the threat of an international outbreak, but would not prevent it (Davis *et al*., 2014).

2.5.1 *Mycobacterium avium subsp. Paratuberculosis*

M. avium subsp. *paratuberculosis* (MAP) is the aetiological agent of paratuberculosis in ruminants and other animals. Paratuberculosis or Johne's disease in cows is an economically important disease especially in dairy cattle. The disease is of major global importance and the Office International des Epizooties (OIE) classifies it as a List B transmitted disease of socioeconomic and/or public health importance. Johne's disease is chronic infectious enteritis caused by MAP. For some time now, MAP has been suspected of involvement in chronic inflammatory changes in the human gastrointestinal tract leading to Crohn's disease, and more recently, there has been a suggestion of its involvement in Type 1 diabetes mellitus (Dow, 2006). Although considered to be autoimmune in origin, there is increasing evidence that Crohn's disease may have an infectious cause (Greenstein, 2003). The pathology of both diseases is similar; however, evidence remains inconclusive regarding a definitive link. If MAP has a role in the pathogenesis of Crohn's disease, then it is possible that infection may arise through food or water (Grant, 2005). In a recent case-control study in the UK, 218 Crohn's disease patients assessed, results could not conclusively support a role for water or dairy

contaminated with MAP in the aetiology of the disease (Abubakar *et al.*, 2007). MAP has been cultured from the milk of clinically and sub-clinically infected bovines (O'Reilly *et al.*, 2004). The organism can also be cultured from the milk of other ruminants, including sheep and goats, affected by Johne's disease. The route of contamination can arise directly either from MAP infected milk in the udder or from faecal contamination during milking. The organism is not reproducibly eliminated by standard pasteurisation (Grant *et al*., 1998).

2.5.2 *Listeria monocytogenes*

This is a gram-positive aerobic non-spore-forming bacterium. Although rare, listerial contamination of dairy products can cause serious illness. These bacteria can thrive in refrigeration temperatures (4°C) and can lead to listeriosis, bacteremia, meningitis, and death for fetuses, children, the elderly, and the immune-compromised. Baek *et al*. (2000) reported that in a survey of food products in South Korea, 4.4% of raw milk products were contaminated with *Listeria* species genetic material, while none were found in pasteurized milk and cheese. This study also mentions that *Listeria* species have been found in pasteurized milk in other countries, for example 1.1% of samples in a United Kingdom survey, but that these were likely due to post-pasteurization contamination. Mathew and Ryser (2002) investigated growth of *Listeria* bacteria that was artificially added into raw and pasteurized milk. The authors found the bacteria were much less likely to grow in raw milk, possibly because of the competing microflora. Another study reported similar results, where four different strains of *Listeria monocytogenes* were artificially incubated in raw or pasteurized milk for 24 hours at 4°C (Pricope-Ciolacu *et al*., 2013). These strains displayed improved virulence when incubated in pasteurized milk, and decreased virulence when incubated in raw milk. These results indicate that the milk environment can impact the virulence of this pathogen, and underscores the importance of preventing post-pasteurization contamination.

2.5.3 *Escherichia coli*

This is a gram-negative bacterium commonly found in the intestines of birds and mammals. Only a small subset of this group of bacteria is pathogenic to humans (e.g. *E. coli* strain O157). For European children under the age of 3, this strain of *E. coli* has caused illnesses solely from drinking raw milk (Baars, 2013). While pasteurization will kill all *E. coli* bacteria, Peng *et al*. (2013) investigated whether subpasteurization, or "thermization", would still be effective in order to retain the claimed health benefits of raw milk. The authors found that thermization did not kill all *E. coli*, but no pathogenic *E. coli* survived. Alhelfi *et al*. (2012) showed that contaminated milk, whether raw or pasteurized, will see proliferation of *E. coli* O157 if allowed to reach room temperature for 2 hours, reemphasizing the need to properly store milk at refrigeration temperatures. Massa *et al*. (1999) also found that storing contaminated raw milk at 8°C, for 1-2 weeks allows *E. coli* O157 to survive and even proliferate.

2.5.4. *Campylobacter jejuni*

This is a gram-negative bacterium that is ubiquitous throughout the environment. They can be present in milk due to faecal contamination during milking or through mastitis in udders. These bacteria can cause campylobacteriosis and in some cases Guillain-Barré syndrome. Doyle and Roman (1982) inoculated *C. jejuni* bacteria into unpasteurized and pasteurized milk. The authors found that *C. jejuni* bacteria levels decreased more rapidly in unpasteurized milk than pasteurized, most likely due to competing microflora. The authors do note the need to pasteurize milk, as *C. jejuni* can be found in unprocessed milk.

2.5.5. *Yersinia entercolitica*

This can grow at refrigeration temperatures. Although they are usually not a concern, they can cause gastroenteritis in susceptible populations such as children. Soltan-Dallal *et al.* (2004) found that 1.6% of raw milk samples from northern Iran tested positive for *Y. entercolitica* genetic material while none of the HTST (high temperature/short time) pasteurized milk samples tested positive. The investigators recognized that other studies have found these bacteria in pasteurized milk samples, but this was usually a result of post-pasteurization contamination.

2.5.6. *Helicobacter pylori*

This is a common parasite infection in humans, usually acquired during childhood from a variety of sources including drinking water and unpasteurized sheep's milk. Fujimura *et al*. (2002) collected bovine milk samples across Japan and found 72.2% of raw bovine milk and 55% of pasteurized milk contained genetic material for the parasite. However, investigators could only isolate live *H. pylori* in one raw milk sample. The investigators concluded that *H. pylori* could not survive pasteurization, but that post-pasteurization contamination is possible.

2.5.7. *Staphylococcus aureus*

This bacterium causes a large number of human infections and can be found throughout the environment. Food handlers and animals can act as reservoirs, and the bacteria can cause mastitis in cows. Rodriguez-Rubio *et al*. (2013) assessed the effectiveness of exogenous lytic enzymes to act as antimicrobials on these bacteria in milk. They found the enzyme CHAPSH3b was particularly effective at destroying these bacteria, more so in raw milk than pasteurized milk. The investigators concluded this was because high temperatures destroyed CHAPSH3b

and thus recommended that the enzyme only be included after pasteurization of milk was complete.

2.5.8. *Arcobacter* species

These are considered emerging enteropathogens, with *A. butzleri* being the most prevalent. These bacteria produce similar symptoms to Campylobacteriosis but are more persistent in the natural environment. Giacometti *et al*. (2014) studied growth and survival of *A. butzleri* and *A. cryaerophilus* that were added "post-processing" to raw, pasteurized, and UHT milk and were then stored for six days. They found at refrigeration temperatures that both species remained viable in all types of milk. At room temperature, *A. butzleri* levels increased in pasteurized and UHT (ultra-high-temperature) milk but became non-viable in raw milk. The authors note that this decrease of these bacteria in raw milk was likely due to competition from other microflora. However, since storing milk at room temperature is never recommended these findings are not relevant. The authors concluded that contamination is mostly a concern during "post-pasteurization" as effective pasteurization will likely remove most if not all *Arcobacter* species.

2.5.9 *Aeromonas*

These bacteria cause gastroenteritis, and are commonly isolated from a variety of food products. These species are able to grow at refrigeration temperatures, thus posing a threat to human health if present in milk. Melas *et al*. (1999) tested many raw and pasteurized milk samples from Northern Greece, and found that 40% of raw milk samples were positive for live *Aeromonas* bacteria, including *A. hydrophila*, *A. caviae*, and *A. sobria*. *Aeromonas* species were not detected in any pasteurized milk samples.

2.5.10 *Coxiella burnetti*

These are found worldwide and can cause an illness commonly referred to as "Q fever". While these bacteria are mostly a hazard for individuals in direct contact with farm animals, there is some concern about exposure through raw milk. However the CDC (Centre for Disease Control and Prevention) considers this exposure rare. Eldin *et al*. (2013) tested raw, thermized, and pasteurized milk for presence of *C. burnetti* genetic material and then tested potential cultures in mice via oral exposure. There were significantly more raw milk samples with the bacteria's genetic material, although some pasteurized milk still tested positive. However none of the mice in the study displayed any illness. The authors consider that pasteurization likely kills *C. burnetti* but may not completely remove its harmless genetic material.

Certain types of bacteria are able to form endospores, a dormant state where bacteria are resistant to extreme conditions such as heat. Endospore-forming bacteria include *Bacillus*, *Paenibacillus* (Scheldeman *et al.*, 2004; ; Huck *et al*., 2007; De Jonghe *et al*., 2010), and *Clostridium botulinum* (Lindstrom *et al*., 2010). The bacteria genus *Bacillus* contains several pathogenic species. De Jonghe *et al*. (2010) detected heat-resistant toxins from *B. amyloliquefaciens* and *B. subtilis* in raw milk, which can cause food poisoning. Banyko and Vyletelova (2009) found similar concentrations of *B. cereus* and *B. licheniformis* in raw and pasteurized milk, and based on genetic fingerprinting determined that most contamination is occurring at points post-pasteurization for pasteurized milk. Huck *et al.* (2007) isolated some of the same strains of *Bacillus* and *Paenibacillus* bacteria in both pasteurized and raw milk, suggesting that these bacteria are not killed during HTST (High Temperature/Short Time) pasteurization. Some *Paenibacillus* strains have even been isolated from UHT-pasteurized (Ultra-High-Temperature) milk (Scheldeman *et al.,* 2004). There is a growing concern that milk, due to its wide distribution, storage in bulk tanks, rapid shelf life, and high consumption rates among humans, could be a prime target for bioterrorist attacks. Newkirk *et al*. (2011)

discusses this topic at length and mentions the potential for very potent pathogens such as *Clostridium botulinum* bacteria, which produce both endospores and deadly neurotoxins, to be used as a weapon. While these bacteria are not commonly found in milk, there are concerns they could be intentionally introduced as part of a bioterrorist plot. Weingart *et al*. (2010) found that HTST (High Temperature/Short time) pasteurization of raw milk removed 99.99% of isolated botulism neurotoxins and 99.5% of the neurotoxin complexes, the latter being the more dangerous form. Perdue *et al*. (2003) grappled with the possibility of an anthrax attack on the milk production system and showed Anthrax is an infection spread by endospores from *Bacillis anthracis*.

2.6 Hygiene, Handling and Microbial Quality of Raw Milk

Milk is a perishable product and an ideal medium for the growth of a wide variety of bacteria (Parekh & Subhash, 2008). When it is secreted from a healthy udder, raw milk contains only a very few bacteria of about 500 to 1,000 bacteria per millilitre (Omore *et al.*, 2005; Pandey & Voskuil, 2011). After milking environmental contamination occurs, which in turns increases the total bacteria count up to 50,000 per ml or may even reach several millions bacteria per milliliter (Pandey & Voskuil, 2011). That count level indicates a very poor hygienic standard of milk during milking and handling or milk of a diseased animal. The presence of coliform bacteria particularly *E. coli* in raw milk is an indicator of feacal contamination which implies poor hygienic conditions and un-sanitized environment since these bacteria are of faecal origin. In developing countries like Nigeria, most of the milk is produced by smallholder farmers dominated by local herds of cattle (Pandey & Voskuil, 2011). Their milking units are widely distributed throughout in rural areas with a poor infrastructure, while most of the markets and customers are in urban areas. Therefore, the need for good hygienic practices and a streamlined collection, handling and transport system is important but has been always a challenge (Pandey & Voskuil, 2011). However, milk contains a natural inhibitory system or temporary germicidal

or bacteriostatic properties which prevents a significant rise in the bacteria count during the first 2 - 3 hours (Swai & Schoonman, 2011; Pandey & Voskuil, 2011). If the milk is cooled to 4ºC within this period immediately after milking, it maintains nearly its original quality and remains safe for processing and consumption. Temperature of storage and time since milking are also important in determining milk quality, as these influence the rate at which the bacteria will increase in number (Omore *et al.*, 2005). To prevent a too high multiplication of bacteria, the milk has to be produced as hygienic as possible and should be cooled or pasteurized immediately (Pandey & Voskuil, 2011).

2.7 Prevention and Control of Microbial Contamination in Milk

Prevention and control of microbial quality of milk is through elimination of organisms from human carriers by general improvements in water supplies, public health education, personal and environmental hygiene. Also, it can be achieved through proper boiling or pasteurization of raw milk before processing and consumption. Pathogenic organisms from the lactating animals can be controlled through improvements in animal husbandry and maintenance of good animal practices, and those from the environments and equipments can be prevented by adhering to general hygienic practices and environmental cleanliness.

Generally, microbial contamination in milk can be minimized through adherence to effective good hygienic practices at farm level; and in order to protect the public against milk-borne infections it is important to screen milk which is informally taken to the market. The lack of awareness of milk-borne infections in many developing countries and consumption of raw milk predispose small-scale livestock keepers, consumers and the general public at risk of contracting these infections (Mosalagae *et al.*, 2011).

2.8 Factors Affecting the Quality of Raw Milk

2.8.1 Physicochemical Properties of Milk

Milk and milk products are excellent high-quality foods providing both nutritional and culinary values (Anderson *et al.,* 2011). This is due to the fact that milk does have distinct physical, chemical and biological characteristics and its colour, odour, taste, consistency, freezing point (-0.55°C), pH (6.6) and specific gravity (1,032) and these characteristics remain particularly constant (Lues *et al.,* 2010). The addition of any foreign material will disturb the milk content hence causing loss to processors and it is cheating to consumers of milk and their products because the consumer needs a quality product for the paid price.

2.8.2 Type of Feed and Cattle Breed

The type of feed given to animals as well as the breed of cattle, they have an effect on both milk composition and milk yield. It is well known that Holstein breeds have the highest milk yield than other breeds but this milk has the lowest fat content (3.64%), and Jersey has the highest fat content (4.64%). This is contributed by interests shown by the breeders with some on the yield and others on milk composition. Nutrition has been reported to affect the milk yield and composition with the increased dry matter intake contributing to increased milk yield and composition of the animal (Agena *et al.,* 2003).

2.8.3 Intentional and Accidental Adulteration of Milk

Adulteration of milk refers to the addition of foreign matter such as flour, margarine and water into the milk (Karimuribo *et al*., 2015). Adulteration of milk can negatively affect its microbial quality, taste and market value (Omore *et al.,* 2005). The adulteration may be done intentionally or accidentally. Accidental milk adulteration might be caused by leaking in the cooling facilities or use of milk containers that are not properly cleaned.

Adulteration is not acceptable because it causes milk contamination; disqualify milk processing into other dairy products by lowering butterfat and protein content. It is not fair to the consumer because they are not receiving the quality product for the price paid. Adulterated milk leads to a loss for processors and consumer. Because milk goes from the farm to the consumer without being tested for quality, the risk of accidental or intentional contamination with foreign matter can be high and go unnoticed.

Intentional adulteration with water and starch can be done to increase the volume or alter the properties of milk. The most common milk adulteration is the addition of water. Adulteration can also be done by adding other substances such as starch, flour and margarine. Water is added with the intention of increasing the volume of the milk (Karimuribo *et al.*, 2015). Starch, flour and margarine are added so as to increase the density and butterfat content of the milk (Karimuribo *et al.*, 2015).

There is little information on the quality of milk consumed in most parts of Tanzania. In a study done by (Orregård, 2013) in Kenya, results showed increased levels of adulteration as the milk was moved from the farmer to the consumer. This creates a need to find out the extent of milk adulteration problem in Hai District Tanzania.

2.8.4 Antibiotic Residues in Milk

Although milk is such an important source of nutrition to people in different age categories, it sometimes contains an unacceptable high level of antibiotic residues so causes problems to consumers of such milk and its products (Muhammad, 2014). Antibiotic residues in milk originate from various sources namely residue of herbicides on feedstuffs, drugs given to cow orally, by injection or as an intramammary infusion for the treatment of mastitis (Jahed, 2007). This problem has led to the development of various techniques to check the level of antibiotic residues in milk to name few DelvoTest and CharmEZ methods. Drug residues in milk apart

from other hazardous effects it also affects negatively the health of the consumer of milk with high level of antibiotic residues. These effects include allergic reactions and bacterial resistance in the body of humans (Muhammad, 2014).

There are ongoing studies aiming at developing the baseline information to help in Hazard Analysis and Critical Control Point implementation to ensure the improvement of the milk quality produced in the developing countries (Grimaud *et al.,* 2009). The HACCP (Hazard Analysis and Critical Control point) approach has an important role in preventing and controlling of chemical contamination in milk and dairy products especially antibiotics in raw milk transported from the producer (Jahed, 2007). The HACCP (Hazard Analysis and Critical Control point) concept is dealing with hazard and risk identification, process decomposition, designation of critical control points, documentation and verification of the programme, is an alternative to the ISO (International Organization for Standardization) system (Lievaart *et al.,* 2005) so as to ensure standardised qualified products. This programme starts at the very early stage of production for dairy products all the way to when the product reaches consumer's hands. The critical control points are defined by the producers depending on the structure of their production units.

The use of drugs in dairy cattle farms is common, being used for treatment as well as feed additives (Navrátilová, 2006; Karimuribo *et al.,* 2015). The frequent use of antibiotics may result in drug residues that can be found at different concentration levels in products from animal origin, such as milk or meat (Khaskheli *et al.,* 2008). In lactating cows, antimicrobial agents are used mostly for the therapy of mastitis but also of other diseases such as laminitis, respiratory diseases, metritis; a foul-fetid vulvar discharge (Navrátilová, 2006). Also on other farms they use antibiotics as prophylaxis i.e giving animals' low dosage of the drug so as to prevent animals from falling sick (Abebew, 2008).

Mastitis is also the problem for dairy cattle in Tanzania. This forces dairy farmers in Tanzania to use antibiotics to treat their animals against this disease as well as other diseases (Mdegela *et al.*, 2009). The common drugs used in dairy farming are β-Lactams such as penicillin, Tetracyclines such as Oxytetracycline and Sulphonamides such as Sulfamethazine (Jahed, 2007). A study done by Navrátilová *et al*. (2009) in the Czech Republic finds a low level of Tetracycline residues in milk. Residues of these drugs in milk may be caused by overdosing the animal or changing the route of administration. An example is giving Fluxinin intramuscularly instead of intravenously and lack of adherence to the withdrawal period (National Milk Producers Federation, 2011). In most developing countries record keeping for animals is not well practised (Orregård, 2013). This creates a need for rapid methods for testing the presence of antibiotics in raw milk to guarantee its quality (Kivirand *et al.*, 2015).

2.9 *Nunu* production process

The production process of *Nunu* in Nigeria is illustrated in Figure 2.1. Typically, fresh cow milk is collected in the morning in calabashes, sieved and left to ferment for a minimum of 24 hours or a maximum of 48 hours depending on the season. During the hot season which is usually from March to June, high ambient temperatures of 35^0C promote acidification of the milk within 12–24 hours yielding the desired product, while in the cold season (October to February) where temperature of 15-17^0C are recorded, the fermentation takes up to 48 hours.

Sometimes, in the dry season when there is not enough grass for grazing of the animals, smaller quantities of milk are obtained and these are added to the previous day's batch depicting a form of backslopping. The fermentation occurs spontaneously without starter cultures and at ambient temperatures. The fermented milk is then churned using a wooden ladle. Fat accumulates as a result of the churning and is removed. Excess whey is drained off to obtain a product with a thick consistency which is the *nunu*, consumed alone or with *fura*

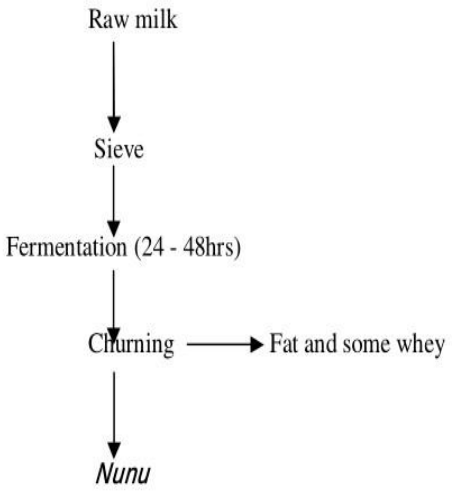


Fig 2.1: *Nunu* production process

Source: Akabanda *et al*., 2014

2.10 Measures to reduce bacterial contamination of raw and market milks

2.10.1 Cleaning and sanitation

In order to reduce/eliminate contamination by spoilage and pathogenic organisms from the farm to the dairy plant, the cow's teats and surrounding udder area, and all utensils and equipment used during milking and processing should be properly cleaned. In a study of bacteria in pasteurised milk, Craven and Macauley (1992) concluded that the standard of hygiene was a major factor contributing to differences in the quality of milk produced by different milk processors. They also suggested that the degree of sanitation and cleaning may influence the type of *pseudomonads*, which contaminate milk (Craven & Macauley, 1992).

2.10.2 Cooling the milk during storage

Rapid cooling of milk after collection is paramount, since contamination of the product with psychrotrophic bacteria is unavoidable. Storage of raw milk at 2^0C has been shown to be effective for shelf life extension compared with storage at 4 and 7^0C (Muir, 1996; Haryani *et al.*, 2003). Furthermore, the storage temperature of raw milk will influence the quality of the

resulting products; the shelf life of UHT (Ultra-high-temperature) milk is much longer when processed from raw milk stored at 2^0C than when processed from raw milk stored at 6^0C (Griffiths *et al.,* 1988). The initial population level has an important influence on growth rates. The higher the initial contamination level of milk, the smaller effect low temperature has on limiting bacterial growth (Champagne *et al*., 1994).

2.10.3 Addition of carbon (IV) oxide

Carbon(IV) oxide (CO_2) at a concentration of 20–30 mM can be added as a preservative in milk. When the air in a sealed container is replaced with CO_2, the HCO_3^- ion is produced, which has antimicrobial properties against psychrotrophic, lactic acid and coliform bacteria (Champagne *et al*., 1994). CO_2 may influence enzyme synthesis also. Sorhaug and Stepaniak (1997) reported that carbon dioxide and nitrogen reduce proteinase secretion at low temperatures.

2.10.4 Thermal treatments

A range of thermal treatments is used to reduce the bacterial population of milk. These include thermisation, batch and HTST (High temperature/short time) pasteurisation, high temperature pasteurisation, UHT (Ultra-high-temperature) treatment and in-container sterilisation (Kelly *et al.,* 2005).

2.10.5 Non-thermal treatments

Several non-thermal treatments can be used to destroy or remove microorganisms in foods (Datta & Deeth, 2002). These include high-pressure treatment, pulsed electric field technology, ultrasonication, centrifugation and microfiltration; however, only the last two technologies are used commercially for milk. Multi-target attack/integrative approaches Integration of several hurdles in the preservation of milk and milk products has been found to be a promising approach because different hurdles may simultaneously and/or synergistically act on different targets (e.g. cell membrane, DNA, enzyme system and other cellular functions) within the

microbial cells so that survival and cell repair would become more difficult (Leistner, 2000). Such approaches may be achieved by combining the effects of antimicrobials and/or other physical treatments. The use of two or more bio-preservatives to yield a synergistic effect on the target organisms has been extensively investigated as means to prolong the shelf life of raw and market milks (Ross *et al*., 2003). Practically, this synergism not only allows very low doses of the antimicrobials to be used effectively, but also expands the range of organisms that may be inhibited; that is organisms normally resistant to each component of the mixture when used separately can also be usefully controlled (Leistner, 2000). Another approach, which has recently received a growing interest from the dairy industry, is the use of natural antimicrobials with other non-thermal preservation methods, including high hydrostatic pressure, pulse electric fields, ultrasonication and irradiation as a potential pathogen intervention strategy for raw and market milks. The synergistic action of such combination preservation systems may offer several advantages such as improvement of the rate of inactivation, development of cost-effective mild preservation, and reduction of the commercial problems associated with sub-lethal injury and survivor tails (Ross *et al*., 2003).

Likewise, it is now being realised that the effect of bio-preservatives is pronounced when combined with certain physical processes (e.g. mild heating, chilling, freezing, drying or homogenisation) because microbial cells sub-lethally injured by such treatments may become more susceptible to the antimicrobials to which the healthy cells are resistant. This approach may be of economic consideration to the dairy industry, not only because of the better image of the products but also because of the reduced processing costs. An interesting instance of such treatments is the activation of the LPO (Lactoperoxidase) systems in milk either before or after heat treatment to reduce the *D* values and to increase the keeping quality of the product (Barrett *et al*., 1999). Similarly, pulsed electric field technology has been combined with pasteurisation to extend the shelf life of milk to up to 78 days (Sepulveda *et al.*, 2005).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Sample Size Determination

A formula by Kothari, (2004) for finite population was used to calculate the sample size for this study;

i.e. $n = \frac{Z^2.p.q.N}{e(N-1)+Z^2.p.q}$

Where Z= 1.96 (desired confidence level at 95% and value obtained from table)

P= 0.5 (Sample proportion according to Kothari (2004), in which case 'N' will be the maximum and the sample will yield the desired precision).

q= 1-p

e= 0.03 (precision rate or acceptable error)

N= 50

$n = \frac{1.96^2x0.5x0.5x50}{0.03(50-1)+1.96^2x0.5x0.5}$

= 19.75; approximately 20samples.

Based on the above formula, 20 samples were collected from each district.

3.2 Sample Collection

The samples were collected from Owerri, Imo State; Elele, Rivers state; Asaba, Delta state and Enugu. The sample collection in Owerri covered the informal mammy and cattle markets at Obinze, and Ama-Hausa in Owerri, Imo State. Also, sample collection at Elele covered the Army Barracks mammy market; in Asaba covered the mammy market and cattle market at Oko, and in Enugu covered the Enugu Express way mammy market. The study areas are dominated by small scale traders, livestock traders and significant number of Fulani settlers.

A total of 100 samples comprising of 20 samples from each location were collected and analysed. These samples were collected between 9.00-11.00am and were transported in an iced flask to the laboratory for immediate analysis.

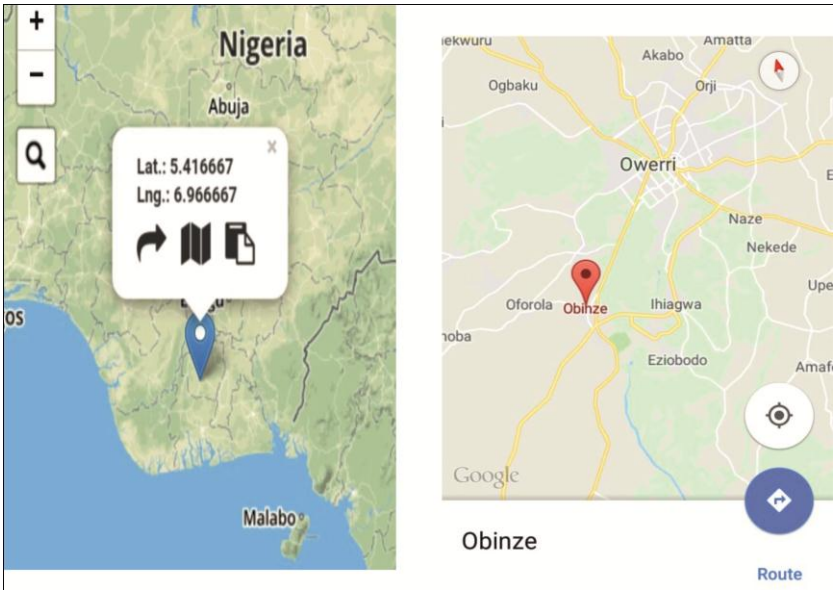


Fig 3.1 Obinze, Owerri west Local Government Area, Imo state, Nigeria.

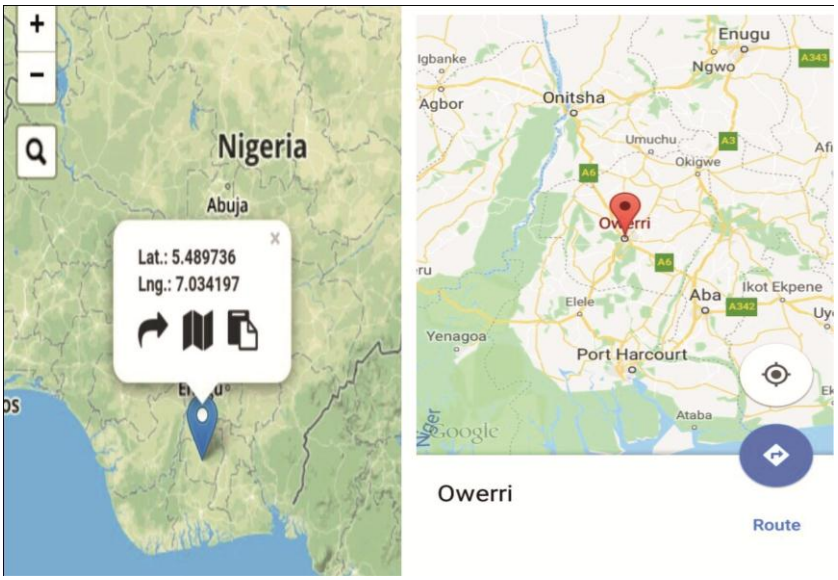


Fig 3.2 Owerri Municipal, Imo state, Nigeria.

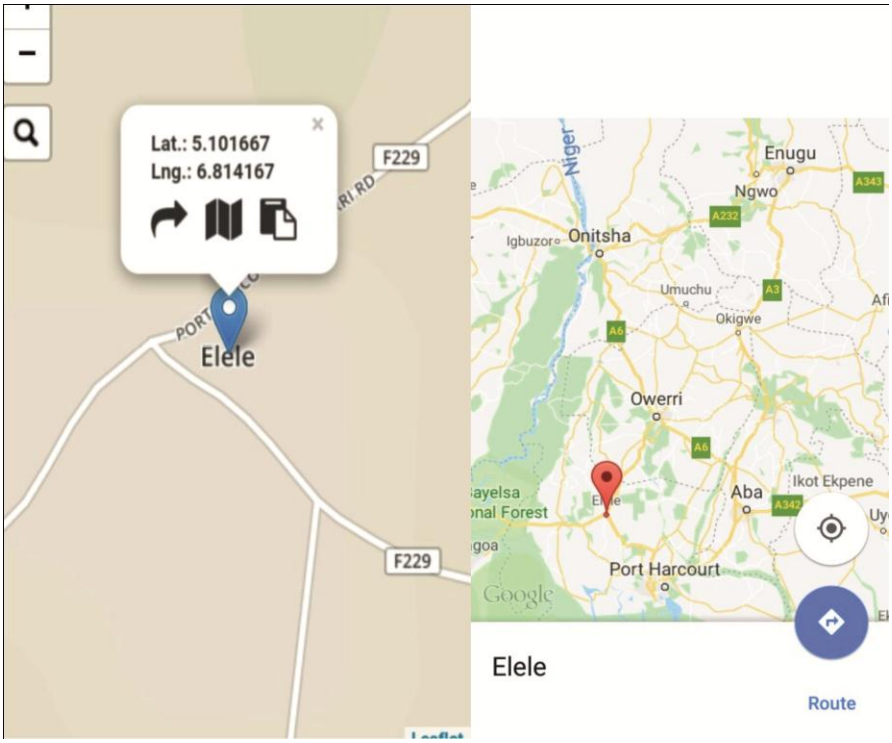


Fig 3.3 Elele, Rivers Sate, Nigeria.

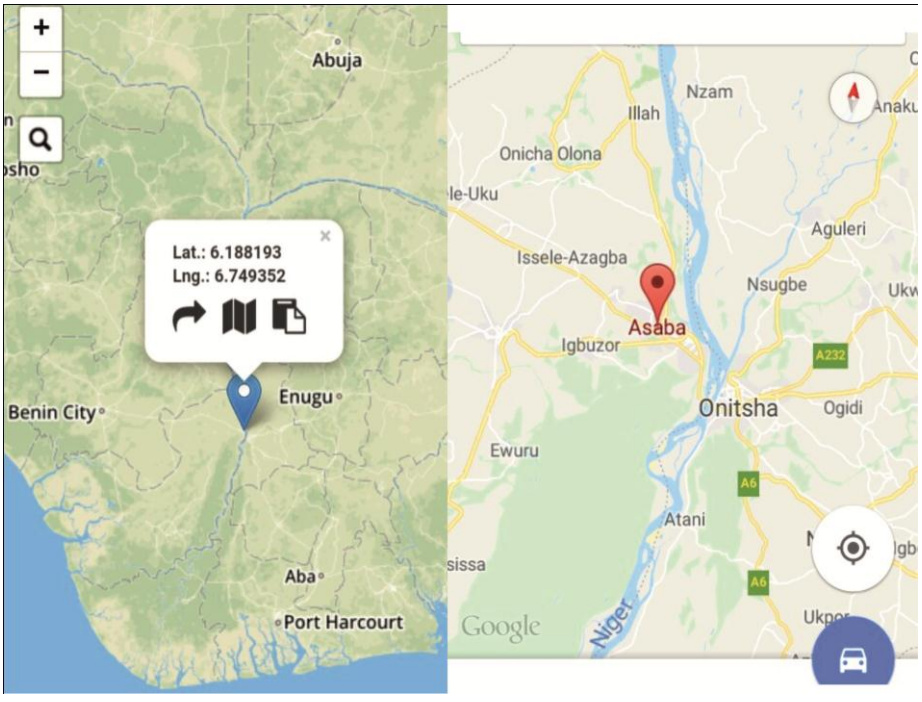


Fig 3.4 Asaba, Delta State, Nigeria.

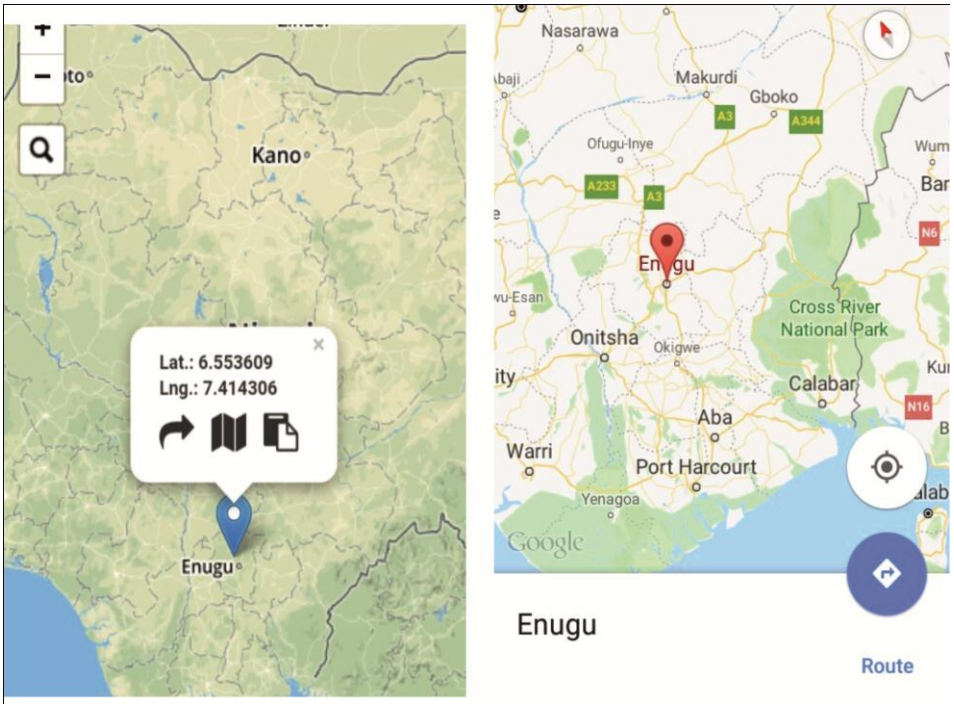


Fig 3.5 Enugu, Enugu State, Nigeria.

3.3 Microbial Analysis

Ten-fold serial dilution of the fermented cow milk was prepared aseptically by dispensing 1ml of each of the milk samples into a test tube containing 9ml sterile distilled water and homogenized by shaking followed by further decimal dilutions to up to 10^{-6} concentrations. One ml of dilutions was dispensed into appropriately labelled sterile Petri dishes with the appropriate media poured into each plate and gently swirled for proper mixture of the medium and sample. The plates were allowed to solidify before incubated at 37^{o}C for 24 hours. Microbial count was determined using direct plate count method as described by (Cheesbrough, 2003).

Media employed for the isolation and enumeration of the organisms include: Nutrient Agar medium for Total Bacteria Count, Eosin Methylene Blue Agar medium for Coliform count, *Campylobacter* blood-free selective Agar for enumeration of *Campylobacter*, Mannitol Salt

Agar for enumeration of *Staphylococcus* sp and *Salmonella-shigella* Agar for enumeration of *Salmonella* sp.

All calculations of colony forming units were done using the formula:

$$\textbf{CFU/ml} = \frac{\textbf{Number of colony counted}}{\textbf{Volume of inoculums}} \times \frac{\textbf{1}}{\textbf{df}}$$

Where; df = dilution factor

3.4 Biochemical identification of the isolates

The representative bacteria colonies that developed on the culture plates were obtained. The various isolates were subjected to gram staining procedure, standard biochemical and physiological test were carried out. References were also made to stock cultures and different microbiology monographs in addition to colonial morphology in order to make proper identifications of the microbial isolate. The biochemical tests for the identification of the isolates were carried out using Cheesbrough, (2003) procedures. Various tests include: Coagulate, catalase, indole, urease production, citrate utilization, methyl red, Voges Proskauer, Kligler Iron, nitrate reduction, glucose, sucrose, lactose, maltose and xylose.

3.5 Determination of Physicochemical and proximate content of *Nunu* samples

3.5.1 Temperature and pH

The temperature of the *nunu* samples was determined at the collection point using thermometer while the pH of the samples were determined in the laboratory using a digital pH meter as described by Okek*e et al.* (2016).

3.5.2 Methylene blue test (MBT)

One ml of methylene blue solution was added to 10 ml of the samples after thorough mixing. The test tubes were sealed with a clean, sterile dipper stopper and slowly inverted each tube twice, to mix the sample and solution thoroughly. The tubes were placed in the water bath at a temperature of 36 °C and examined the samples after 30 minutes. Then the final time of colour

disappearance was recorded throughout sample tests (Ombui *et al.,* 1995 ; Worku *et al.,* 2012; Braide *et al*., 2015).

3.5.3 Resazurin test (RT)

The samples were mixed thoroughly and 10 ml was poured into previously sterilized test tube. One ml of resazurin solution (working solution) was added quickly into the test tube. The samples were placed in the water bath at 37°C and reading was taken at hourly intervals. After the first hour, tubes was examined by noting the degree of colour change from blue through mauve, purple, pink and finally colourless after a stated period of incubation, or the time required reducing the dye to a predetermined colour (Benson, 2002; Braide *et al*., 2015).

3.5.4 Titrable Acidity test

Nine militers of the sample was mixed with 1ml of phenolphthalein in a conical flask and then slow addition of 0.1N sodium hydroxide with a burette. The end point was pink colour appearance (National Centre for Excellence in Mathematics and Science Teaching and learning, 2015).

$$\%\text{Lactic acid} = \frac{ml \times M \times 90 \times 100}{V \times 1000}$$

ml = ml of 0.1N NaOH

M= Molarity of NaOH

V = ml of Sample solution used

90 = MW of Lactic acid

3.5.5 Alcohol Test

Five millilitre of sample was transferred to a test tube and rapidly add 5ml of 70-75% ethanol solution. The content was mixed by inverting the tube twice. I observed for coagulation, clothing or precipitation (Sharma, 2005).

3.5.6 Determination of Alkaline Phosphatase activity

This was done using the procedures stated by University of Vermont (2001).

A. Standard Curve Determination

1. Using serial dilution techniques, 1mL of four p-nitrophenol concentrations, ranging from 0.8mg/ml to 0.1mg/ml was prepared.

2. Using the stock p-nitrophenol solution and the 0.05 M Citrate Buffer to make the dilutions in 4 marked test tubes. Taking 0.5 mL of each dilution made in step 1, and adds it to 4.5 mL of 0.02 M NaOH and mix properly.

3. Setting the wavelength of the spectrophotometer to 410 nm and blank with a cuvette of 0.02M NaOH.

4. Transferring the dilutions (prepared in no. 2) to a cuvette one at a time (enough to fill) and the absorbance of each at 410 nm was measured.

5. Graph of absorbance data against the concentration of the solutions measured was made.

B. Standard Phosphatase Reaction Assay

This is a discontinuous enzyme assay. The reaction occurs for a total of 10 minutes, which two absorbance reading was taken.

1. Two “stop test tubes” of 4.5 mL of 0.02 M NaOH was prepared. This strong base will halt the enzyme reaction by denaturing the enzyme, and turn the product yellow. The reaction was stopped 2 times.

2. Starting the reaction. The reaction mixture is made of three parts:

a. 1.5 mL of 0.05 M Citrate Buffer, pH 4.8

b. 1.5 mL of 1mM p-nitrophenyl phosphate (substrate)

c. 1.5 mL of 1 mg/mL phosphatase (Sample)

The buffer and substrate was added to a clean test tube. Then, the sample was added to initiate the reaction, the tube was quickly and briefly stirred (vortex), and the stopwatch was started.

3. The reaction was run for a total of 10 minutes, 0.5 mL of the mixture from the "reaction tube" was taking with pipette into one of the 2 "stop tubes" containing 4.5 mL of 0.02 M NaOH.
4. Three minutes after adding an aliquot of the reaction to a "stop tube," cuvette was filled with this second mixture. The stopped mixture's absorbance in a spectrophotometer at 410 nm was measured.

3.5.7 Determination of Heavy metals in *nunu* samples

The heavy metals were determined using Atomic Aborption Spectrometry as described by Ahmed *et al.* (2017).

Acid digestion of cattle's milk: All glassware were first cleaned with 10% HNO_3 solution and then further washed with the distilled water. Milk of 10 mL concentration was digested with 1:3 of H_2O_2 and HNO_3 on a hot plate. The samples were heated on hot plate until their volume reduces to 2 mL. This 2mL sample solution was then diluted with 20 mL distilled water and make a clear solution of it. The contents of the beaker brought to the required volume with distilled water and were examined by Flame Atomic Absorption Spectrophotometer.

Preparation of standards of heavy metals: The heavy metals selected for study were Cd, Zn, and Pb. In each case of the selected metals, three different concentrations were made to calibrate the Flame AAS. These concentrations are as follows: 1.0 ppm, 1.5 ppm and 2.0 ppm. The resultant calibration curve of well-prepared standard concentrations gives linear curve by Atomic Absorption Spectrophotometric Analysis by using Perkin Elmer PinAAcle™ 900T atomic absorption (AA) spectrophotometer (Shelton, CT, USA) which is equipped with the sensitive WinLab32™ for AA software running under Microsoft® Windows™ 7 for flame absorption spectrophotometry.

3.6 Determination of Proximate Composition

3.6.1 Moisture content

Dry Oven method was used as described by Udensi *et al.* (2018). Weighed 10ml of the sample into a pre-dried and pre-weighed crucible and dry in the oven at 105^0C for 2hrs until a constant weight is obtained. Cool the crucible and its content in a dessicator and weighed.

Calculations:

$$\text{Moisture\%} = \frac{W_2 - W}{W_3 - W_2} \times 100$$

Where;

W_1 = weight of empty crucible; W_2 = weight of crucible and sample before drying

W_1 = weight of crucible and sample after drying

3.6.2 Determination of Crude fibre

Crude Fibre was determined as described by Okeke *et al.* (2016). Two mililitres of sample was defatted with petroleum ether, boiled in 100ml of 0.25N H_2SO_4 and filtered. This was washed in boiling water, dried in oven, and weighed.

$$\text{\% of crude fibre} = \frac{W_2 - W}{\text{Weight of sample}} \times 100$$

3.6.3 Determination of crude protein

Total protein content of the nunu samples was determined according to the Kjeldahl method of the Association of Official Analytical Chemists (AOAC) as described by Gemechu *et al*. (2015) and Teklemichael *et al.* (2015). For digestion, 5ml of nono sample was warmed in a water bath at 38^oC and poured into a Kjeldahl flask. A mixture of 15g potassium sulphate, 1ml of copper Sulphate solution and 25ml of concentrated Sulphuric acid was added into the flask and mixed gently. The digestion was carried out in a digestion block until a clear solution appeared. Then, it was allowed to cool at room temperature. The digested solution was diluted

with 250ml of distilled water. For distillation, digestion flask was placed in the distillation equipment. 75ml of 40% sodium hydroxide solution were added into it. Then ammonia was distilled and 50ml of 40% boric acid solution using bromocresol green indicator were added until blue colour appeared. Finally, the sample was titrated with 0.1N hydrochloric acid solution from a burette until a faint pink colour solution was formed and the burette reading was taken to the nearest 0.01ml. Blank test was carried out using the above procedure except that water was used instead of test sample.

The percentage of nitrogen in the *Nunu* samples were calculated as follows:

$$N\ (\%) = \frac{(Vs-Vb)\ HCl\ consumed \times N \times 1.4007}{Sample\ weight}$$

$$CP\ (\%) = N\ (\%) \times 6.38$$

Where N (%) = Percentage nitrogen by weight, Vs = Volume of HCl used for titration of sample, Vb = Volume of HCl used for titration of the blank, CP (%) = Percentage of crude protein, N= Normality of HCl used.

3.6.4 Determination of fat content

Five grams of samples were mixed with methanol and chloroform. This was then centrifuged. The chloroform layer was removed and the fat residue weighed (Okeke *et al*., 2016).

% fat = weight of fat/Weight of sample x100 (AOAC, 2000).

3.6.5 Determination of Ash Content

The ash content of the *nunu* samples was determined gravimetrically according to Dafur *et al.* (2018). The dried *nunu* samples used for determination of total solids content were ignited in a muffle furnace at a temperature of 550^{o}C until they were free from carbon (heating continued until black colour disappeared or the ash residue appears greyish to white) for four hours, and then the samples were transferred to the desiccators to cool down. Finally, the ash content was calculated according as:

$$\text{Ash\%} = \frac{\text{Residue weight}}{\text{Weight of sample}} \times 100$$

3.6.6 Carbohydrate determination

Diference method was used to calculate the percentage carbohydrate as described by Udensi *et al.* (2018).

Carbohydrate % = 100 – (Ash + crude protein+ moisture+ fat+ fibre) %

3.7 Statistical Analysis

Mean and standard deviation of sample values were done using Excel (2007). Analysis of variance was done using Excel (2007) and Post hoc analysis done using Minitab version 17. All Microbial results analysed were transformed to $\log_{10}$ values using Past statistical software. Similarity in distribution of bacterial isolates in the locations was done using Sorenson's Coefficient.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Results

4.1.1 Microbiological Analysis of *Nunu* samples

The total bacterial count of *Nunu* samples in different locations and mean total bacterial count (log_{10} CFU/ml) of 20 samples each of five different locations is shown in Fig 4.1. The mean total bacterial count was calculated by converting the microbial count to log_{10} Values, and taking the average for the different locations. The maximum value recorded was $log_{10}9.669$ taken from Army Barracks mammy market; Obinze and the minimum value recorded was $Log_{10}9.542$ taken from Enugu Express way mammy market.

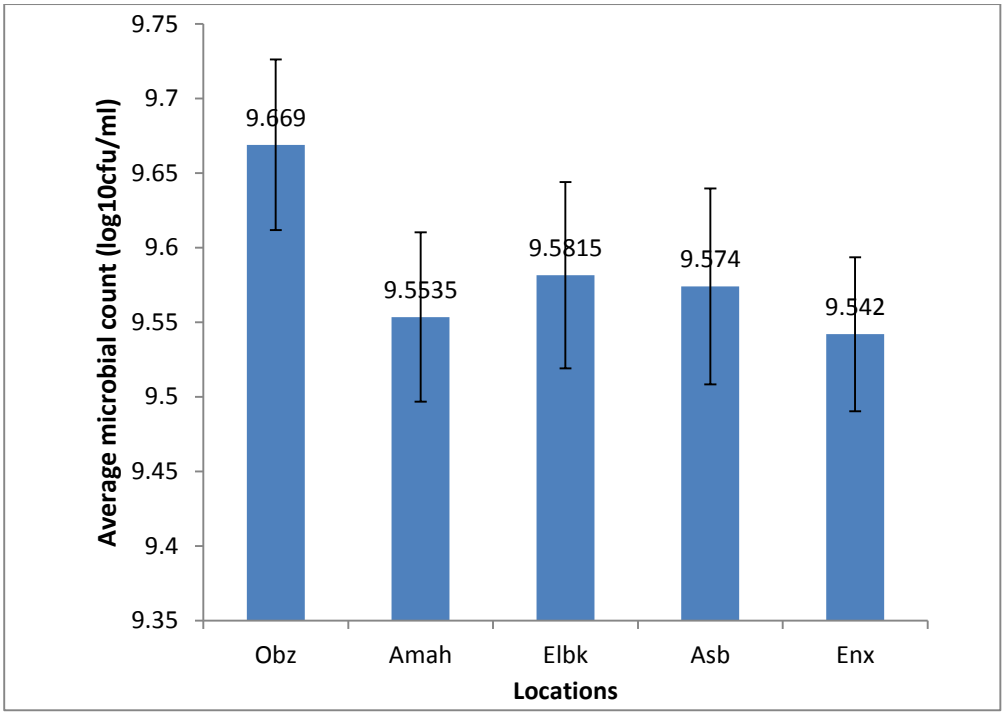


Fig 4.1: Average total Bacterial count of 20 samples each of five different locations

Values = mean±std; n=20; P>0.05

Key: Elbk=Elele Army Barracks mammy market; **Amah**= Amah Hausa market, Owerri; **Obz**= Obinze Army Barracks mammy market; **Enx**, Enugu Express way mammy market; **Asb**, Asaba mammy market;

The average total *Campylobacter* count (Log_{10} CFU/ml) of five different samples locations and mean total *Salmonella* count (Log_{10} CFU/ml) of five different sample locations are shown in Fig 4.2 and Fig 4.3 respectively. Samples taken from Obinze had the highest mean *Campylobacter* count and seconded by Elelle samples with and Log_{10}4.887CFU/ml and Log_{10}4.441 CFU/ml respectively. Samples taken from Elelle had the highest mean *Salmonella* count and seconded by Obinze samples with and Log_{10}2.552CFU/ml and Log_{10}2.535CFU/ml respectively. While Samples taken from Asaba had the least mean *Salmonella* count with 1.28Log_{10}CFU/ml and Enugu samples had least mean *Campylobacter* count with 3.487Log_{10}CFU/ml.

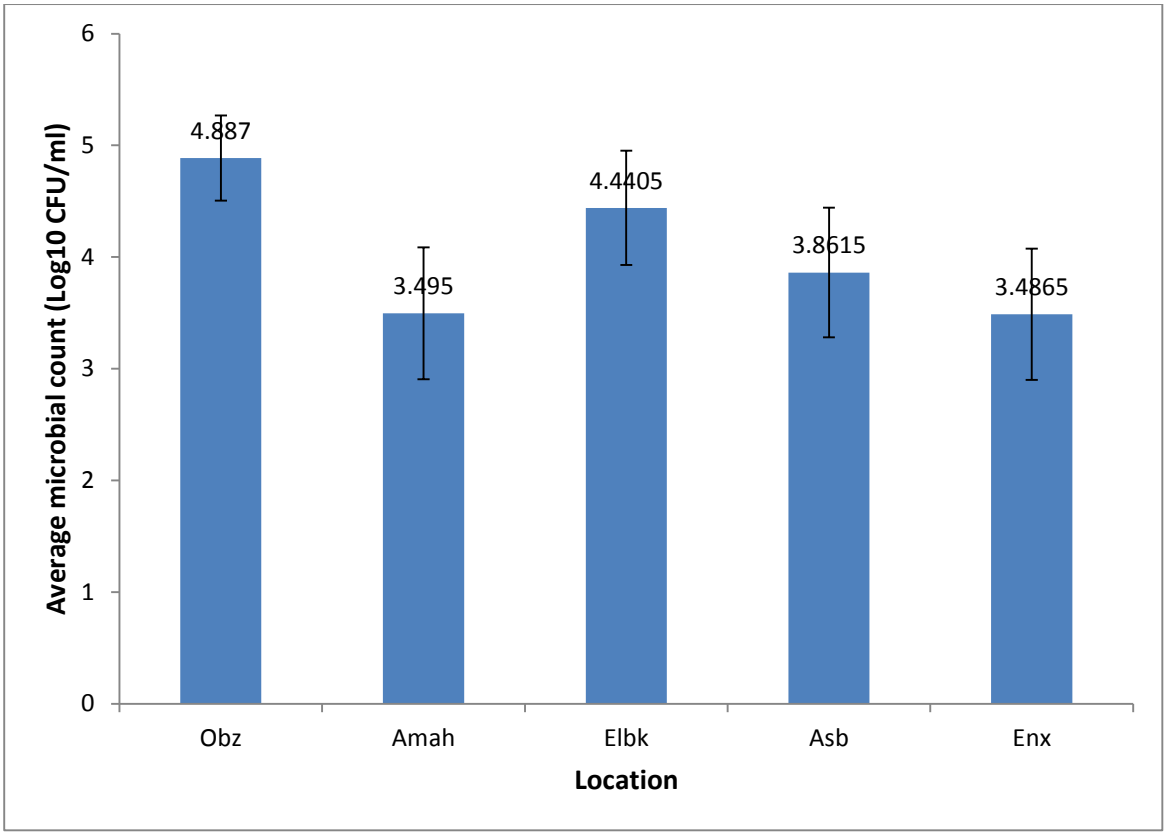


Fig4.2: Average Total *Campylobacter* count (log_{10} cfu/ml) of five different sample Locations

Values = mean±std; n=20. P>0.05

Key: Elbk=Elele Army Barracks mammy market; **Amah**= Amah Hausa market, Owerri; **Obz**= Obinze Army Barracks mammy market; **Enx**, Enugu Express way mammy market; **Asb**, Asaba mammy market;

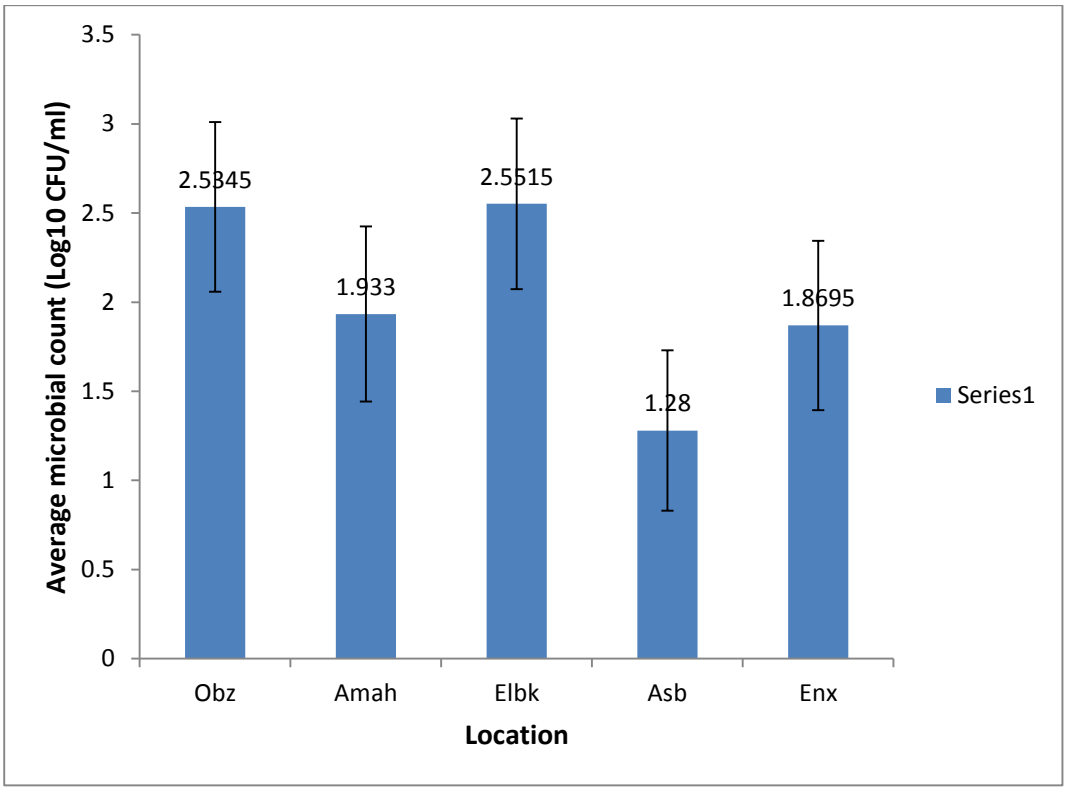


Fig 4.3: Average Total *Salmonella* count (log_{10} cfu/ml) of five different sample Location

Values = mean±std; n=20. P>0.05

Key: Elbk=Elele Army Barracks mammy market; **Amah**= Amah Hausa market, Owerri; **Obz**= Obinze Army Barracks mammy market; **Enx**, Enugu Express way mammy market; **Asb**, Asaba mammy market;

The mean total *Staphylococcal* count ((log_{10} CFU/ml) and mean total Coliform count (log_{10} CFU/ml) of five different sample locations are shown in Fig 4.4 and Fig 4.5 respectively. The samples taken from Elele had the highest mean *Staphylococcal* count with 5.044Log_{10}CFU/ml, while Obinze samples had the highest mean Total Coliform count with 4.191Log_{10}CFU/ml. Asaba samples had the least mean total *Staphylococcal* count and mean total Coliform count with 3.36Log_{10}CFU/ml and 1.837Log_{10}CFU/ml respectively.

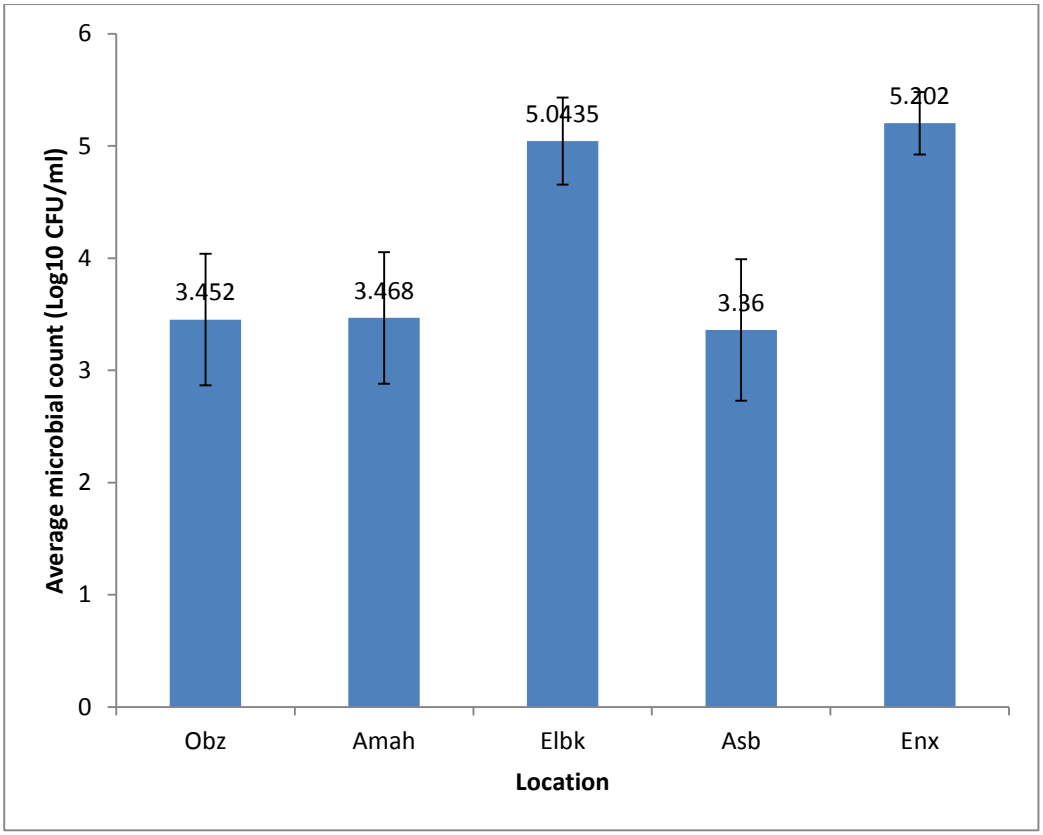


Fig4.4: Average Total Staphyloccocal count (log_{10} cfu/ml) of five different sample locations

Values = mean±std; n=20. P<0.05

Key: Elbk=Elele Army Barracks mammy market; **Amah**= Amah Hausa market, Owerri; **Obz**= Obinze Army Barracks mammy market; **Enx**, Enugu Express way mammy market; **Asb**, Asaba mammy market;

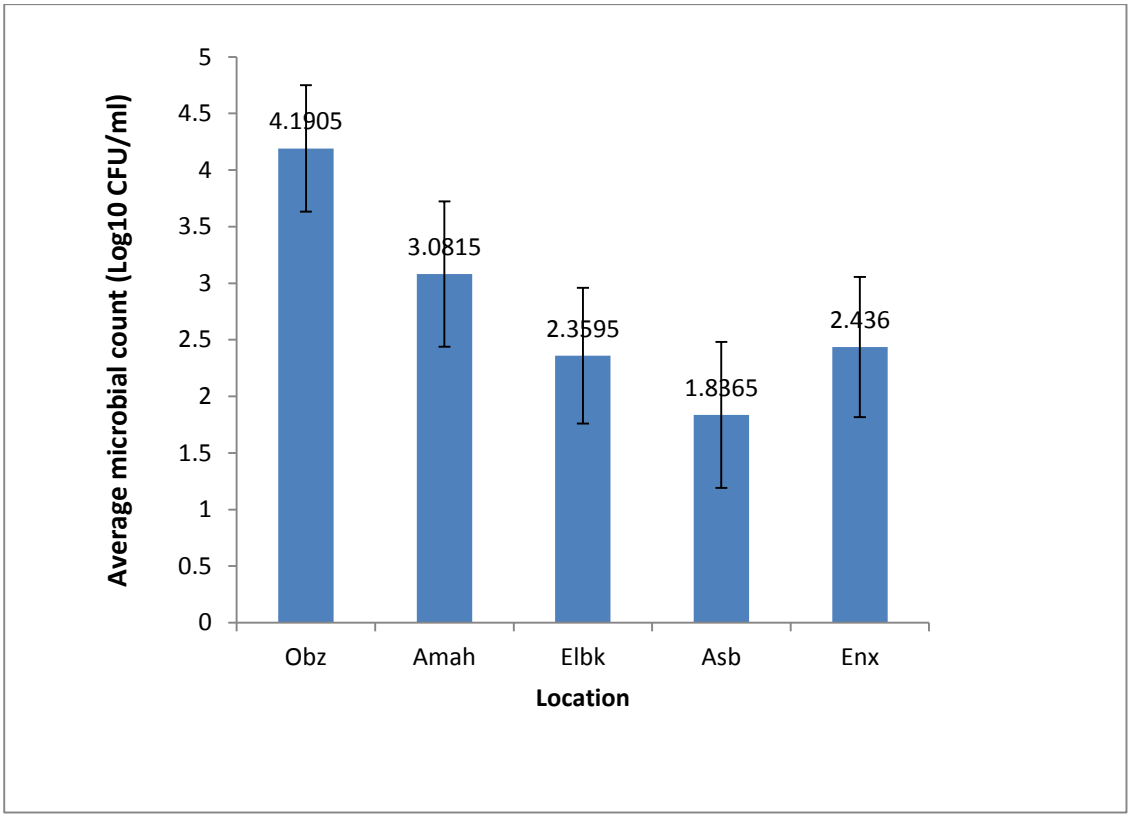


Fig 4.5: Average Total Coliform count (log_{10} cfu/ml) of five different sample locations

Values = mean±std; n=20. P>0.05

Key: Elbk=Elele Army Barracks mammy market; **Amah**= Amah Hausa market, Owerri; **Obz**= Obinze Army Barracks mammy market; **Enx**, Enugu Express way mammy market; **Asb**, Asaba mammy market;

4.1.2 Biochemical identification of the isolates

The Tables 4.1 and 4.2; Fig 4.6 and Table 4.3 show colonial and microscopic characteristics of Bacteria isolated from *Nunu* samples, Biochemical and Carbohydrate fermentation test of bacteria isolated from *Nunu* samples, Percentage occurrence of bacterial isolates and distribution of bacterial isolates in the samples from five different locations respectively.

Enterococcus sp showed the highest percentage occurrence with 28.7% and E. coli showed lowest percentage occurrence with 3.27%. *Enterococcus* appeared in all samples from the five locations. The community similarity done using Soreenson's Coefficient showed a high similarity between the locations.

Table 4.1: colonial and Microscopic characteristics of Bacteria isolated from *Nunu* samples

Colonial morphology	Gram morphology	Motility	Sporulation	Capsule formation	Most probable identity
Small circular moist and shiny low convex bright yellow colonies on nutrient agar	Gram positive cocci predominantly in tetrads, few in clusters	Non motile	-	-	*Micrococcus* sp
Circular moist and shiny golden yellow colonies on nutrient agar and mannitol salt agar	Gram positive cocci predominantly in clusters, few in pairs and tetrads	Non motile	-	-	*Staphylococcus* sp
Small smooth moist and shiny low convex cream colonies on nutrient agar	Gram positive cocci in chains	Non motile	-	-	*Enterococcus* sp
Small circular moist and shiny low convex orange colonies on nutrient agar	Gram positive cocci predominantly in tetrads, few in clusters	Non motile	-	-	*Micrococcus* sp
Dull and dry serrated flat cream colonies on nutrient agar	Gram positive central spores in short chains	Motile	+	-	*Bacillus* sp
Small smooth golden yellow colonies on campylobacter blood free agar	Gram negative slender rods in short chains	Non motile	-	-	*Campylobacter* sp

Moist and shiny mucoid creamy white colonies on campylobacter blood free agar	Gram negative slender rods in short chains	Non motile	-	-	*Campylobacter* sp
Small smooth light pink colonies on salmonella shigella agar	Short gram negative rods in chains	Non motile	-	-	*Shigella* sp
Shiny black fish eye colonies on salmonella shigella agar	Short slender gram negative rods predominantly in singles	Motile	-	-	*Salmonella* sp
Mucoid and shiny pink colonies on Eosine methylene blue agar	Gram negative rods in short chains and singles	Non motile	-	-	*Enterobacter* sp
Shiny greenish metallic sheen on Eosine methylene blue agar	Gram negative rods predominantly in singles	Motile	-	-	*Escherichia coli*
Mucoid and slimy pink colonies on Eosine methylene blues agar	Large Gram negative rods in chains	Motile	-	+	*Klebsiella* sp
Large raised slimy cream colonies on nutrient agar	Large gram positive rods in short chains	Motile	+	-	*Bacillus* sp

Table 4.2 : Biochemical and Carbohydrate Fermentation Test of Bacteria isolated from *Nunu* samples

CAT	OXI	COAG	IN	MR	VP	Cit	URS	NO_3	GLU	SUC	LAC	MAL	XYL	Identity of isolates
+	-	-	-	+	-	+	+	+	+	-	-	+	+	***Salmonella* sp**
+	-	-	-	-	+	+	+	+	+	+	+	+	+	***Klebsiella* sp**
+	-	-	-	-	+	+	-	-	+	-	+	-	-	***Enterobacter* sp**
+	-	-	-	-	+	-	+	+	+	+	+	+	-	***Staphylococcus aureus***
+	-	-	-	-	+	+	-	+	+	-	-	-	-	***Bacillus cereus***
-	-	-	-	+	-	+	-	-	+	+	+	-	-	***Enterococcus faecalis***
+	-	-	+	+	-	-	-	+	+	+	+	+	+	***Escherichia coli***
+	-	-	-	-	+	+	-	+	+	-	-	+	-	***Bacillus subtilis***
+	-	-	-	-	+	-	-	+	+	-	-	-	+	***Campylobacter* sp**
+	-	-	-	-	+	+	-	+	-	-	-	-	-	***Micrococcus luteus***
+	-	-	-	-	+	+	-	+	+	+	-	-	-	***Micrococcus roseus***
-	-	-	-	+	-	+	-	+	+	+	-	+	-	***Shigella* sp**

CAT, catalase; OXI, oxidase; COAG, coagulase; IN, indole; MR, methyl red; VP, Voges Proskaeur, CIT, citrate, URS, urease production; NO_3^-; nitrate reduction; GLU, glucose;

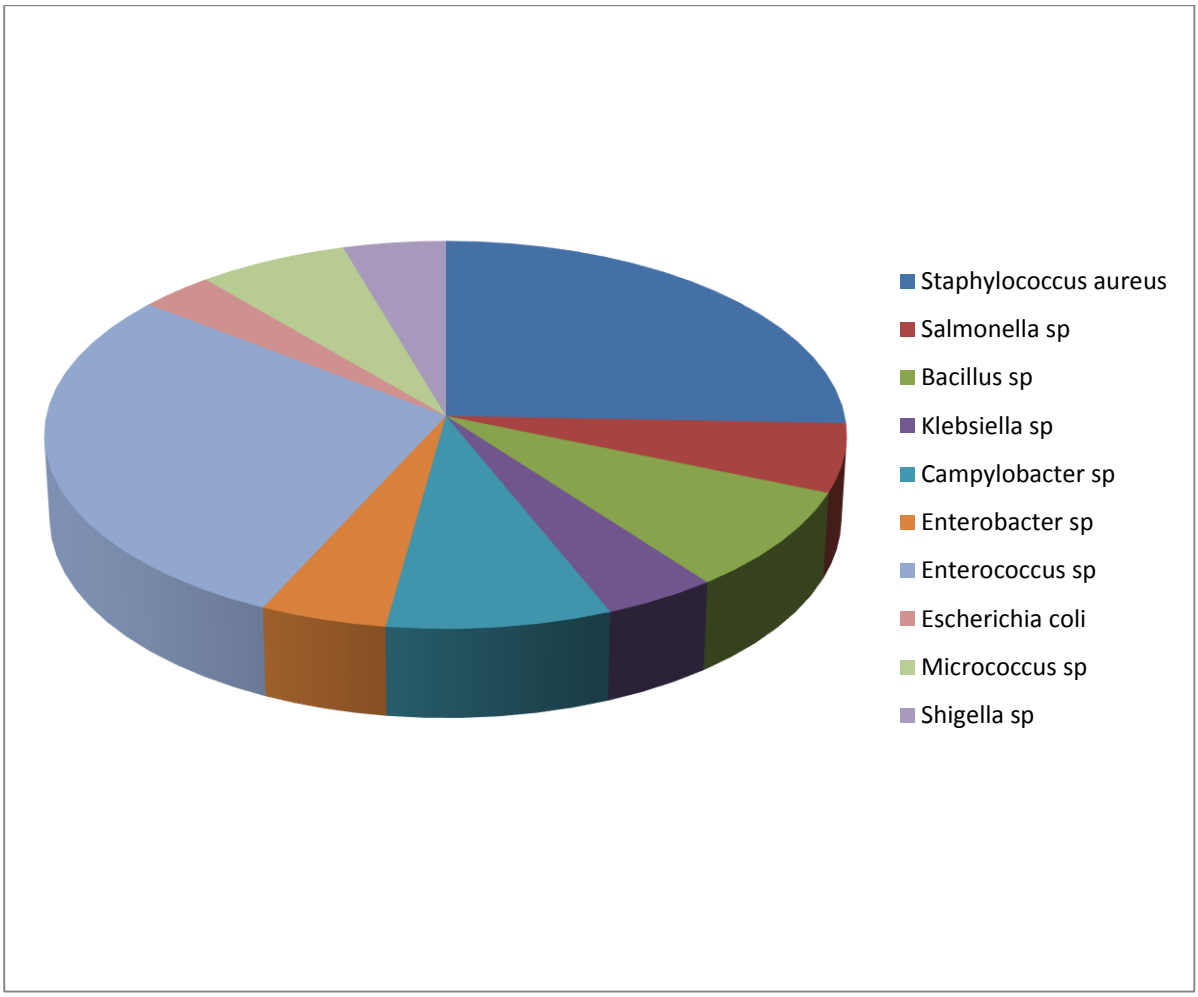


Fig4.6: Percentage occurrence of Bacterial Isolates

Table 4.3: Similarity in Bacterial Distribution in the samples collected from different Locations

Location	Bacteria
Obz^{A}	*Staphylococcus* sp, *Enterococcus* sp, *Bacillus* sp, *Campylobacter* sp, *Enterobacter* sp, *Micrococcus* sp, *Klebsiella* sp, *Shigella* sp, *Escherichia coli*
Amah^{A}	*Staphylococcus* sp, *Salmonella* sp, *Bacillus* sp, *Escherichia coli, Enterobacter* sp, *Bacillus* sp, *Micrococcus* sp, *Enterococcus* sp, *Campylobacter* sp
Elbk^{A}	*Campylobacter* sp, *Bacillus* sp, *Staphylococcus* sp, *Micrococcus* sp, *Escherichia coli, Salmonella* sp
Enx^{A}	*Staphylococcus* sp, *Campylobacter* sp, *Enterobacter* sp, *Escherichia coli, Salmonella* sp,
Asb^{A}	*Campylobacter* sp, *Bacillus* sp, *Staphylococcus* sp, *Micrococcus* sp, *Escherichia coli, Salmonella* sp, *Shigella* sp

Key: Elbk=Elele Army Barracks mammy market; **Amah**= Amah Hausa market, Owerri; **Obz**= Obinze Army Barracks mammy market; **Enx**, Enugu Express way mammy market; **Asb**, Asaba mammy market;

Same superscript alphabet (in the location column) shows there is similarity in bacterial distribution between the locations.

4.1.3 Physicochemical and proximate content of *Nunu* samples

4.1.3.1 pH and Temperature

The pH in this study ranged from 4.69 to 5.23 with no significant difference in the mean pH values of the different sample locations ($p>0.05$). The pH results obtained in this study is presented in fig4.7.

The temperature recorded in this study ranged from 27.47^0C to 27.79^oC with no significant difference between the different sample locations ($p>0.05$). The temperature results obtained in this study is presented in Fig4.8.

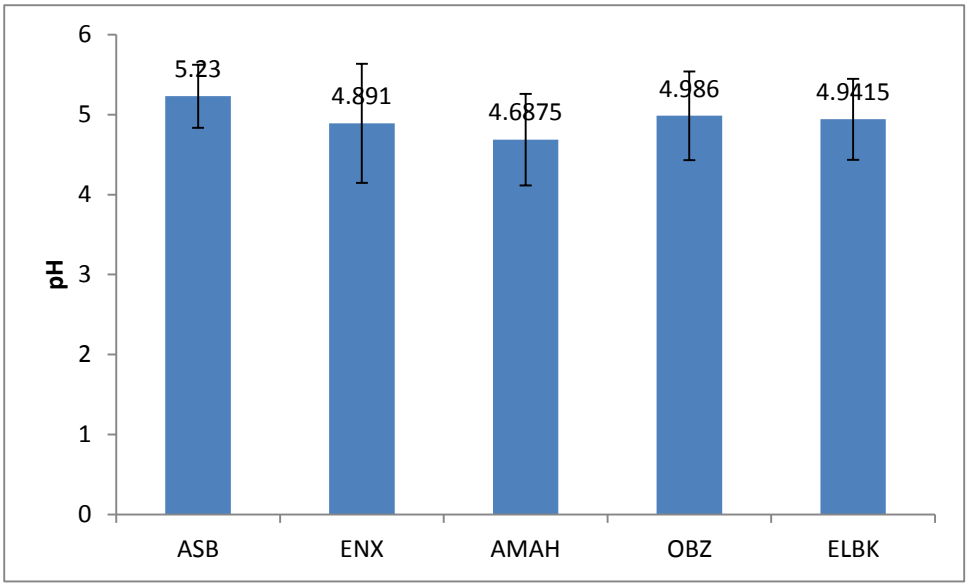


Fig4.7: pH of *Nunu* samples from five different locations.

Values are mean± std; n= 20 for each location; P>0.05
Key: Elbk=Elele Army Barracks mammy market; **Amah**= Amah Hausa market, Owerri; **Obz**= Obinze Army Barracks mammy market; **Enx**, Enugu Express way mammy market; **Asb**, Asaba mammy market;

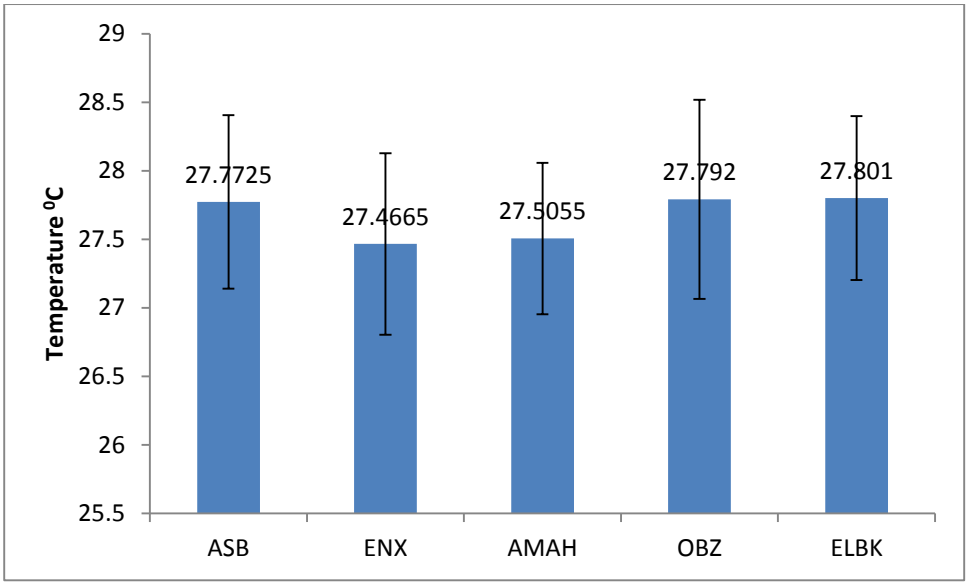


Fig 4.8: Temperature of *Nunu* samples from five different locations.
Values are mean± std; n= 20 for each location; P>0.05

Key: Elbk=Elele Army Barracks mammy market; **Amah**= Amah Hausa market, Owerri; **Obz**= Obinze Army Barracks mammy market; **Enx**, Enugu Express way mammy market; **Asb**, Asaba mammy market;

4.1.3.2 Resazurin test

The result of methylene blue test of 100 samples across five different locations is shown in Table 4.4. Within 30mins of incubation, 30%, 40%, 20%, 40% and 30% of samples from Obinze, Owerri, Elele, Asaba and Enugu respectively change to white. All the samples change to white within 120mins of incubation.

Table 4.4: Resazurin Test on *Nunu* samples from three different Sample Locations.

SAMPLE CODE	TIME (MINS)	COLOUR CHANGE	SAMPLE	MILK QUALITY
Obz	30	WHITE	6	VERY BAD
	60	WHITE	4	VERY BAD
	120	WHITE	10	VERY BAD
Amah	30	WHITE	8	VERY BAD
	60	WHITE	4	VERY BAD
	120	WHITE	8	VERY BAD
Elbk	30	WHITE	4	VERY BAD
	60	WHITE	10	VERY BAD
	120	WHITE	6	VERY BAD
Asb	30	WHITE	8	VERY BAD
	60	WHITE	8	VERY BAD
	120	WHITE	4	VERY BAD
Enx	30	WHITE	6	VERY BAD
	60	WHITE	10	VERY BAD
	120	WHITE	2	VERY BAD
CONTROL	150	PINK	CONTROL	FAIR

N=20 for each location

Key: Elbk=Elele Army Barracks mammy market; **Amah**= Amah Hausa market; **Obz**= Obinze Army Barracks mammy market; **Enx**, Enugu Express way mammy market; **Asb**, Asaba mammy market; Control Sample = from Owerri

4.1.3.3 Methylene blue test

The result of Methylene blue test of 100 samples from five different locations is shown in Table 4.5. Most of the milk (*Nunu)* samples showed very short decolouration time of the dye with minimum time of the blue colour disappearance recorded to be 60mins.

Table 4.5: Methylene blue test of *Nunu* Samples from five Different Sample Locations

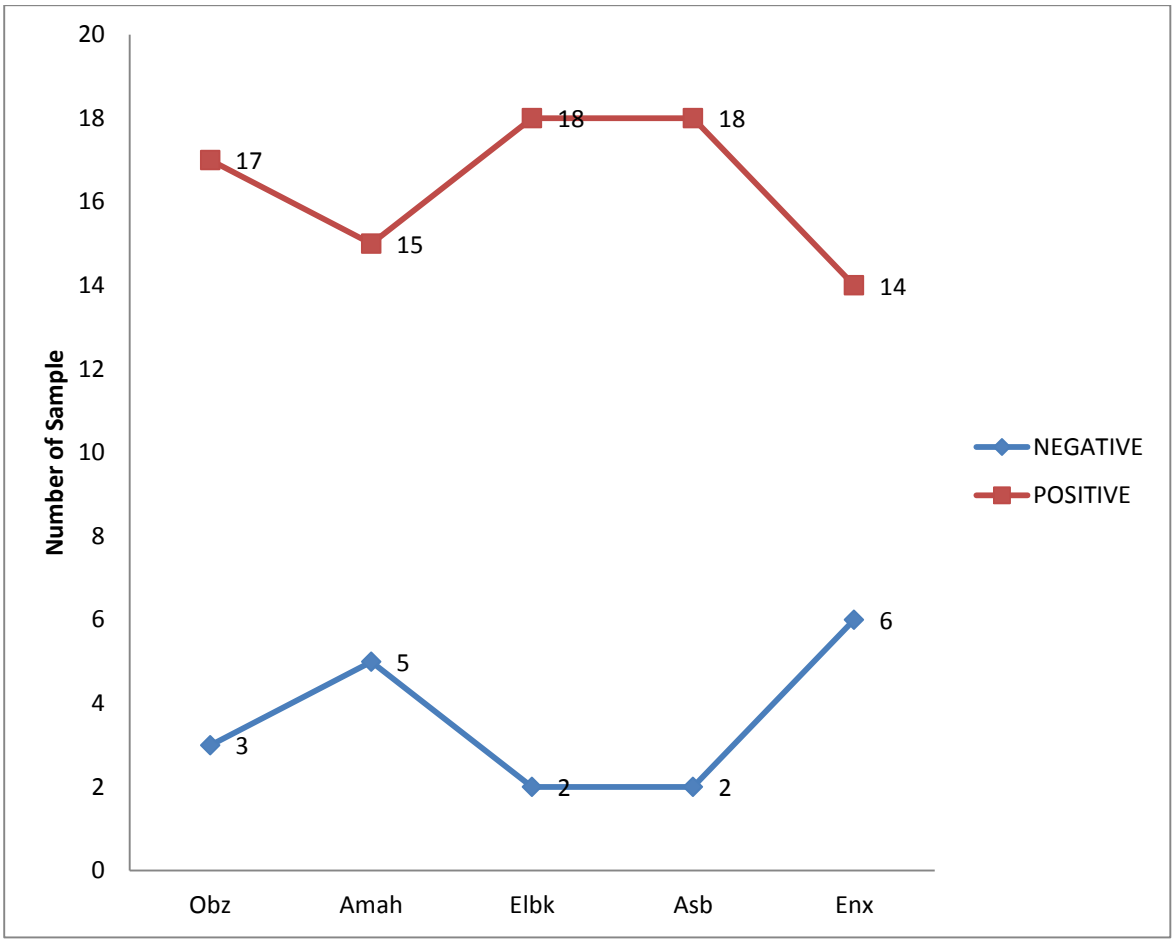
SAMPLE CODE	TIME (MINS)	COLOUR CHANGE	NO. SAMPLE	MILK QUALITY CLASS
Obz	30	BLUE-WHITE	0	-
	60	BLUE-WHITE	8	POOR
	120	BLUE-WHITE	10	POOR
	150	BLUE-WHITE	2	FAIR
Amah	30	BLUE-WHITE	0	-
	60	BLUE-WHITE	9	POOR
	120	BLUE-WHITE	8	POOR
	150	BLUE-WHITE	3	FAIR
Elbk	30	BLUE-WHITE	0	-
	60	BLUE-WHITE	11	POOR
	120	BLUE-WHITE	7	POOR
	150	BLUE-WHITE	2	FAIR
Asb	30	BLUE-WHITE	0	-
	60	BLUE-WHITE	12	POOR
	120	BLUE-WHITE	7	POOR
	150	BLUE-WHITE	1	FAIR
Enx	30	BLUE-WHITE	0	-
	60	BLUE-WHITE	8	POOR
	120	BLUE-WHITE	9	POOR
	150	BLUE-WHITE	3	FAIR
CONTROL	270	BLUE-WHITE	CONTROL	GOOD

N=20 for each location

Key: Elbk=Elele Army Barracks mammy market; **Amah**= Amah Hausa market; **Obz**= Obinze Army Barracks mammy market; **Enx**, Enugu Express way mammy market; **Asb**, Asaba mammy market; Control Sample = from Owerri

4.1.3.4 Alcohol Test

The alcohol test of the milk samples (*Nunu*) from different locations is shown in **Fig 4.9**. Alcohol test is a rapid test for the determination of milk acidity. In this result, 87% of the total samples (100) showed positive result and only 13% of the total samples across all locations showed negative result.



N=20 for each location

Key: Elbk=Elele Army Barracks mammy market; **Amah**= Amah Hausa market, Owerri; **Obz**= Obinze Army Barracks mammy market; **Enx**, Enugu Express way mammy market; **Asb**, Asaba mammy market

Fig 4.9: Alcohol Test of *Nunu* Samples from five Different Sample Locations

4.1.3.5 Titrable Acidity

The titrable acidity of the milk samples (*Nunu*) from different locations is shown in Fig4.10. Titrable acidity is a test to determine the spoilage of milk samples. It quantifies the amount of lactic acid produced by lactic acid bacteria in the samples. The sample from Elelle had the highest mean acidity with 0.745±0.182 %lactic Acid and samples from Asaba had the lowest mean acidity with 0.638±0.158 %lactic Acid

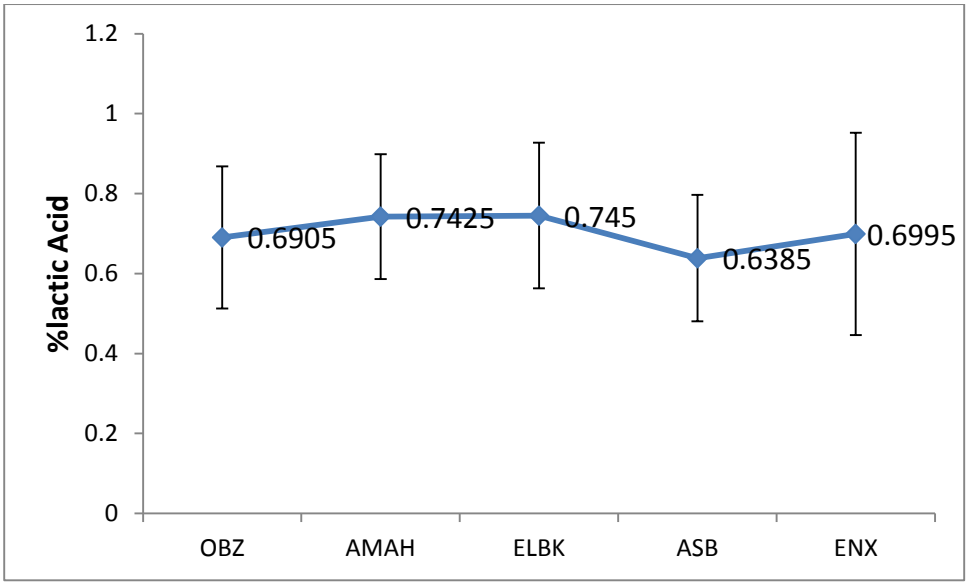


Fig4.10: Titrable Acidity of *Nunu* samples from five Different Sample locations.

Key: Elbk=Elele Army Barracks mammy market; **Amah**= Amah Hausa market, Owerri; **Obz**= Obinze Army Barracks mammy market; **Enx**, Enugu Express way mammy market; **Asb**, Asaba mammy market;

4.1.3.6 Alkaline phosphatase activity

The alkaline phosphate activity in milk (*Nunu*) sample from different locations is shown in Fig 4.12. Detection of enzyme indicates microbial contamination for unpasteurized milk and inadequate pasteurisation for pasteurised milk. The fig 4.11 shows the standard calibration curve of p-nitrophenol plotted with absorbance at 410nm against concentration of the p-nitrophenol. The R^2 value indicates the closeness of the points to the regression line. The R^2 is between 0 to 1, and is regarded as perfect graph. The equation of the graph was y=1.123x where 'y' is the absorbance and x is the concentration of p-nitrophenol of the samples.

The concentration of the p-nitrophenol in the samples ranged from 0.294 to 0.361mg/l. The sample from Elelle had the highest mean p-nitrophenol concentration with 0.361±0.063mg/l and samples from Asaba had the lowest mean p-nitrophenol concentration with 0.294±0.091mg/l.

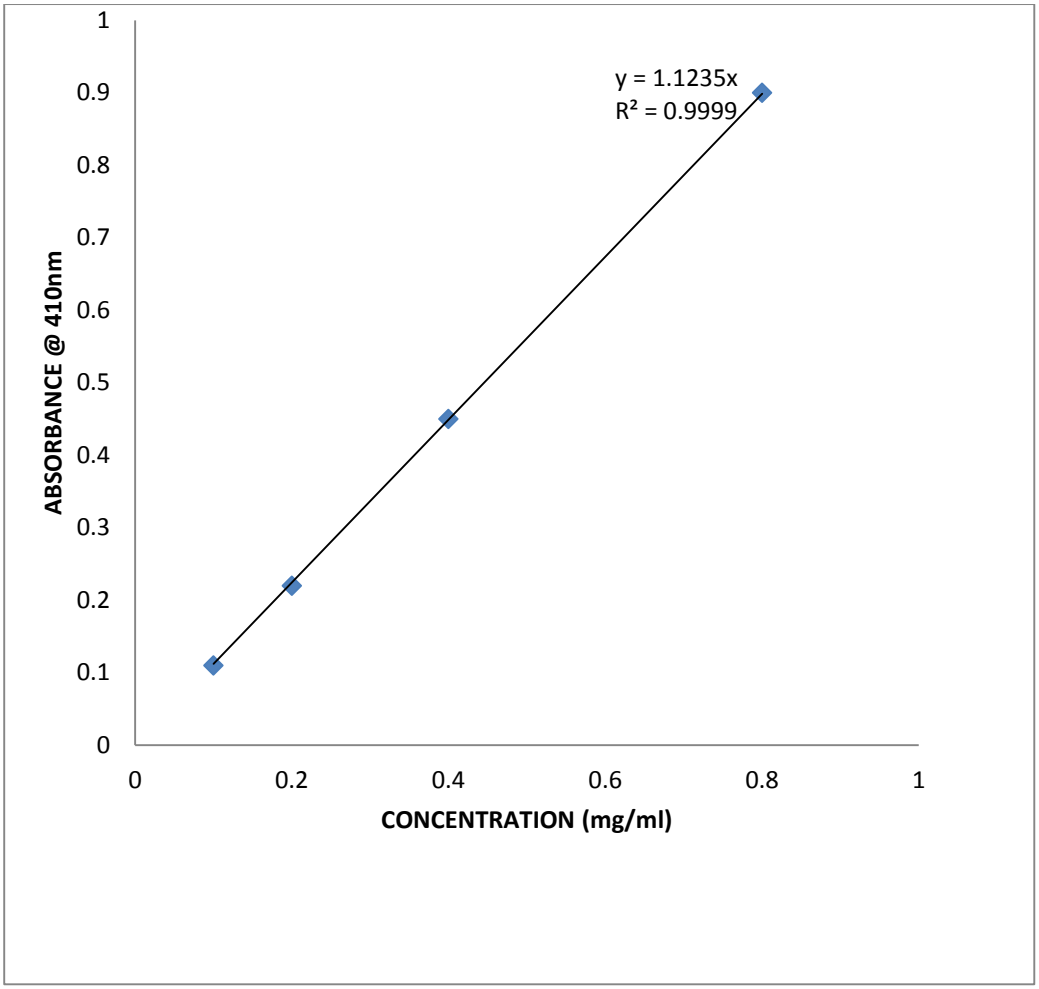


Fig4.11: Standard Curve of p-NitroPhenol

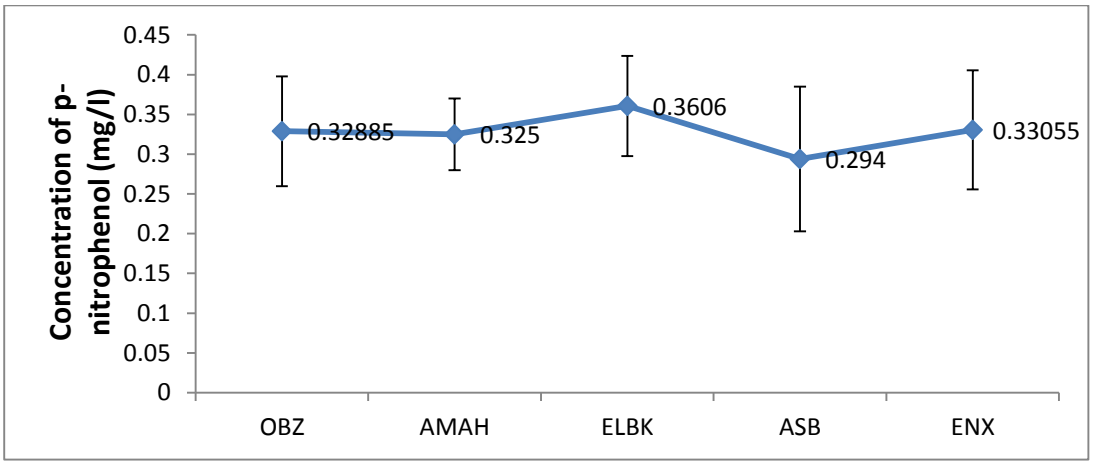


Fig 4.12: Alkaline Phosphotase activity of *Nunu* samples from five different locations.

Values are mean± std; n= 20 for each location; P>0.05

Key: Elbk=Elele Army Barracks mammy market; **Amah**= Amah Hausa market, Owerri; **Obz**= Obinze Army Barracks mammy market; **Enx**, Enugu Express way mammy market; **Asb**, Asaba mammy market;

4.1.3.7 Heavy metal concentrations

The concentration of some heavy metals obtained in this study is presented in Table4.6. The lead Concentration in this study ranged from 0.0033±0.001 to 0.0058 ±0.009 with no significant difference existing between the mean lead concentration in different locations ($p>0.05$).

The Cadmium concentration in this study ranged from 0.0064 to 0.0074 mg/l with no significant difference existing between the mean lead concentration in different locations ($p>0.05$).

The Zinc concentration in this study ranged from 0.615 to 0.805mg/l. There is significant difference existing between the mean zinc concentration in different locations ($p<0.05$). The Tukey pairwise comparison showed that the Asb samples had the highest mean concentration (0.805±0.08mg/l) but not significantly higher than Obz, Amah, and Elbk samples. Meanwhile, Enx samples had the least mean zinc concentration (0.615±0.19mg/l) but not significantly lower than Obz, Amah, and Elbk samples.

Table4.6: Heavy Metal Concentrations of *Nunu* samples from five different locations

Sample Locations	Zn (mg/l)	Cd (mg/l)	Pb (mg/l)
Asb	0.805±0.08[a]	0.0064±0.003[a]	0.0051±0.009[a]
Enx	0.615±0.19[b]	0.0074±0.002[a]	0.0051±0.009[a]
Amah	0.705±0.19[ab]	0.0077±0.003[a]	0.0033±0.001[a]
Obz	0.715±0.16[ab]	0.0070±0.003[a]	0.0053±0.009[a]
Elbk	0.675±0.18[ab]	0.0074±0.004[a]	0.0058±0.009[a]

Values are mean± std; n= 20 for each location,

Values with the same letter in the same column are not significantly different (p>005).

Key: Elbk=Elele Army Barracks mammy market; **Amah**= Amah Hausa market, Owerri; **Obz**= Obinze Army Barracks mammy market; **Enx**, Enugu Express way mammy market; **Asb**, Asaba mammy market;

4.1.4 Proximate Composition of *Nunu* samples

The proximate compositions of the *Nunu* samples are presented in Table4.7. The moisture content is ranged from 80.73 – 85.12%. There is significant difference in the moisture content of the *Nunu* samples from the different locations ($p<0.05$). The Tukey pairwise comparison showed that Asb samples (85.12±0.42%) was significantly higher than other locations, while Amah and Elbk samples had the least mean moisture contents (80.76±0.11 and 80.73±0.16 respectively) with no significant difference between them.

The Fat content is ranged from 3.40 – 3.62%. There is significant difference in the fat content of the *Nunu* samples from the different locations ($p<0.05$). The Tukey pairwise comparison showed that Enx samples (3.62±0.07%) was significantly higher than other locations, while Obz sample had the least mean fat contents (3.40±0.06).

The Ash content is ranged from 1.32 – 0.93%. There is significant difference in the Ash content of the *Nunu* samples from the different locations ($p<0.05$). The Tukey pairwise comparison showed that Obz and Elbk samples (1.32±0.06% and 1.30±0.10% respectively) were significantly higher than other locations with no significant difference between them, while Asb sample had the least mean Ash content(0.93±0.03%).

The Crude Protein content is ranged from 2.34 – 3.34%. There is significant difference in the Crude Protein content of the *Nunu* samples from the different locations ($p<0.05$). The Tukey pairwise comparison showed that Asb samples (3.34±0.16%) was significantly higher than other locations, while Amah, Obz and Elbk samples had the least mean Crude protein contents (2.33±0.07, 2.34±0.08 and 2.28±0.09 respectively) with no significant difference between them.

The Carbohydrate content is ranged from 6.95 – 12.16%. There is significant difference in the carbohydrate content of the *Nunu* samples from the different locations ($p<0.05$). The Tukey pairwise comparison showed that Elbk and Amah samples (12.16±0.05 and 12.16±0.28 %) was significantly higher than other locations with no significant difference between them, while Asb samples had the least mean carbohydrate content (6.95±0.20%).

Table4.7: Proximate Composition of *Nunu* samples from five different locations

SAMPLE	Moisture %	Fat%	Ash%	Fibre%	Crude Protein%	Carbohydrate%
ASB	85.12±0.42^{a}	3.55±0.03^{b}	0.93±0.03^{d}	ND	3.34±0.16^{a}	6.95±0.20^{d}
ENX	83.64±0.30^{b}	3.62±0.07^{a}	0.99±0.01^{c}	ND	3.08±0.28^{b}	8.63±0.06^{c}
AMAH	80.76±0.11^{d}	3.52±0.07^{bc}	1.23±007^{b}	ND	2.33±0.07^{c}	12.16±0.05^{a}
OBZ	81.79±0.28^{c}	3.40±0.06^{d}	1.32±0.06^{a}	ND	2.34±0.08^{c}	11.09±0.10^{b}
ELBK	80.73±0.16^{d}	3.49±0.04^{c}	1.30±0.10^{a}	ND	2.28±0.09^{c}	12.16±0.28^{a}

Values are mean± std; n= 20 for each location, ND= Not detected

Values with the same letter in the same column are not significantly different (p>005)

Key: Elbk=Elele Army Barracks mammy market; **Amah**= Amah Hausa market, Owerri; **Obz**= Obinze Army Barracks mammy market; **Enx**, Enugu Express way mammy market; **Asb**, Asaba mammy market;

4.2 Discussion

4.2.1 Microbiological quality of *Nunu* samples

Total bacterial count was used as important indicator of the microbial quality of the milk sample (*Nunu*). No significant difference ($p>0.05$) exist between the total bacterial count of the samples from different locations. From the results of this study, it was found that all of the milk samples had higher TBC (total bacterial count) than the maximum recommended level of $\log_{10}$ 6.30 CFU/ml (2.0×10^6 CFU/ml) as given by East Africa Community Standards (EAS 67: 2007), and $\log_{10}$ 5.70CFU/ml given by Thai Agricultural Standard (TAS 6003-2010). The mean TBC obtained from this study was higher than that of Briade *et al.* (2015) ($\log_{10}$ 9.64 CFU/ml), Worku *et al.* (2012) ($\log_{10}$7.22 CFU/ml), and Esther *et al.* (2004) ($\log_{10}$ 7.48 CFU/ml). These high counts suggest this *Nunu* unfitness for human consumption. The implication of these results is that *Nunu* from these locations is of poor microbial quality. Presence of high total bacterial load in raw milk indicate contamination possibly from lactating cows, milking equipments, storage containers, unsatisfactory hygiene/sanitation practiced at farm level, unsuitable storage condition, unclean udder/ or teats, poor quality of water used for cleanliness and poor personal hygiene of the milkers (Bukuku, 2013; Kanyeka, 2014).

The mean total *campylobacter* and *salmonella* count of samples from five different locations respectively is shown in Figure 4.2 and 4.3. There was no significant difference ($p> 0.05$) in the total *Salmonell*a count between mean counts of five locations. Obinze samples recorded the highest with $\log_{10}$ 2.53CFU/ml and Asaba samples recorded the least mean value with 1.28Log_{10} cfu/ml. None of the sample locations was able to pass the microbiology guideline given by Centre for Food Safety (CFS) (2014), that n=5; c=0,1,1 and 0; m=0,10,10 and 0; M=0,100,100 and 0 for *Campylobacte*r count, *Staphylococcus,* coliform and *Salmonella* respectively. Where n= number of sample, c= number of positive sample, m= acceptable level

and M= unacceptable level. There was no significant difference ($p<0.05$) in mean total *campylobacter* count between samples from the five different locations with the least mean value from Asaba samples.

The mean total *Staphylococca*l count of samples from five different locations is shown in Fig 4.4. There was significant difference ($p< 0.05$) in mean total *Staphylococcal* count between samples from the five locations, with Enugu and Elele samples both recording the highest count with $\log_{10}$ 5.20CFU/ml and $\log_{10}$ 5.04CFU/ml respectively. However, the detection of *Salmonella* sp, *Staphylococcus* sp and *Campylobacter* sp is in accordance with the report of Chambers (2002) that pathogenic organisms that externally contaminate milk are *Salmonella* and *Campylobacter* strains. According to Chambers (2002), these organisms are from sources external to the udder.

The use of coliform count as an indicator of sanitation has been a common tool in public health protection for many years (Chambers, 2002, Yousef & Carlstrom, 2003). The Fig 4.5 showed the mean total coliform count of five different locations, which was found to be $\log_{10}$ 4.19 CFU/ml for Obinze Army Barracks mammy market, $\log_{10}$ 3.09 CFU/ml for Owerri Amah Hausa market, $\log_{10}$ 2.36 CFU/ml for Elele Army Barracks mammy market; $\log_{10}$ 1.84 CFU/ml for Asaba mammy market; and $\log_{10}$ 2.44 CFU/ml for Enugu Express way mammy market, which was significantly ($p< 0.05$) higher than the maximum recommended level of 10^4CFU/ml (EAS 67:2007, TAS6003-2010). There was no significant difference ($p>0.05$) in the TCC between mean count of the five different locations with Obinze Army Barracks mammy market recording the highest mean TCC with $\log_{10}$ 4.19 CFU/ml and Asaba recorded the least mean TCC with $\log_{10}$ 1.84 CFU/ml. Nevertheless, *E. coli* as the faecal coliforms was detected in all locations with 28% distribution with Obinze samples detected in 6 samples out of 10samples. This is in agreement with reports suggesting high *E. coli* as coliform bacteria in Tanzania (Mdegala *et al*., 2009; Bukuku, 2013).

The morphological, cultural and biological characteristics of microbial isolates (Table 4.1) revealed the following probable bacteria genera; *Micrococcus* sp to *Enterobacter* sp among others. The detection of these bacteria suggest probable faecal contamination and being enteric bacteria indicate poor hygiene practice among handlers (Donkor, 2007), and this corresponds to the high total bacterial count recorded for the five different samples. The detection of these organisms in milk sample was also reported by Egwaikhide *et al*. (2014) in Nigeria. These organisms have been reported to be responsible for the spoilage of milk by different researchers such as Donkor *et al.* (2007), Tamime (2009) and Bukuku, (2013).

The microbiological study of *Nunu*, showed the dominance of *Enterococcus* with 28.7% distribution and were present across the five locations. This was contradictory to the report of Braide *et al*., (2014) that reported *Bacillus* spp to be most distributed in milk sample. In this report, *Bacillus* spp was the 3rd most highly distributed behind *Staphylococcus* and *Enterococcus* spp across the five locations. Although, this study is in accordance with Akabanda *et al*. (2010) that reported Lactic acid bacteria to be dominant species in the fermentation of *Nunu*.

However, the higher values found in this study for Total bacterial count, Total coliform count, Total *Staphylococcal* count, Total *Campylobacter* count and total *Salmonella* count for the *Nunu* samples collected from five locations at selling point compared to EAS and TAS standards could be attributed to the cumulative results of milk contamination at different levels as reported by Karimuribo *et al*. (2005) and Makerere University (2011) which include; insufficient pre-milking udder preparation, insufficient cleaning of milkers hands, and milking utensils use of poor quality and non-boiled water for cleaning of milk equipments and storage containers. Additional handling of milk with different containers may increase the chances of the *Nunu* being more contaminated.

4.2.2 Physicochemical and Proximate Quality of *Nunu* samples

4.2.2.1 pH

The pH in this study ranged from 4.69 – 5.23. The range of pH in this study corresponds to the high titrable acidity observed. The low pH ought to prevent the growth of spoilage organisms and pathogenic organisms according to Omola *et al*. (2019), however Obi and Ikenebomeh (2007) suggested that high microbial growth in fermented raw milk might be due to the initial pH of (6.68) raw milk which is suitable for the growth of heterogenous microbes. The pH obtained in this study is similar to the report of Omola *et al.* (2019) and Tankoano *et al.* (2016). The high bacteria count recorded in this study despite the low pH might be due to the high initial pH of the raw milk before fermentation.

4.2.2.2 Temperature

The temperature in this study ranged from 27.47^0C to 27.79^0C. This result is similar to the report of Dafur *et al.,* (2018). The high temperature observed in this study must have contributed to the high bacteria count observed in this study. The Industrial standard recommends for fermented milk products is temperature $<.8^0$C (Dafur *et al*., 2018).

4.2.2.3 Methylene blue test

This is one of the dye reduction test method use to determine hygienic status of the milk sample. The shorter time required for the disappearance of the blue colour is indicative of a higher microbial load (Worku *et al*., 2012, Braide *et al*., 2015). The suggested milk quality classification is as follows; class1- Excellent (not decolourized in 8hrs), class2- Good (decolourized in less than 8hrs but not less than 6hrs), Class3-fair (decourized in less than 6hr but not less than 2hrs) and class4-poor (decolourized in less than 2hrs) (Braide *et al.,* 2015).

The methylene blue reduction time according to Thai Agricultual Satandard (TAS 6003-2010) shall be longer than 4hrs. In this study, it is shown that all samples changed colour before 4hrs of incubation with the minimum of 60mins and maximum decolouration time of 2hrs30mins as shown in table 4.6. This may be due to poor milk handling practices during milking, poor animal health services, and use of poor potable water which were linked to high mean total bacterial count (Nandy *et al*., 2007).

4.2.2.4 Resazurin Test

This is one of the dye reduction test use to determine the hygienic status of the milk sample (Braide *et al.,* 2015). According to Thai Agricultual Satandard (TAS 6003-2010), the resazurin reduction test shall be at least grade 4.5 at 1hr incubation. At 30mins, 30%, 40%, 20%, 40% and 30% of the samples from Obinze, Owerri, Elelle, Asaba, and Enugu respectively showed colour change to white. Only 50%, 40%,30%, 20%, and 20% of the samples from Obinze, Owerri, Elele, Asaba, and Enugu respectively were able to stay up to 2hrs incubation period before turning to white. This rapid decolouration showed high microbial contamination of the milk samples (Worku *et al*., 2012).

4.2.2.5 Alcohol Test

This has been used for rapid detection of milk acidity (spoilage of milk). Positivity of the result shows the milk is sour and is unfit for any processes involving pasteurisation, because the proteins in milk having increased acidity have also lost the stability for the temperatures used for pasteurisation. From this study, 85% of sample from Obinze, 85% of samples from Owerri, 95% of samples from Elele, 90% of samples from Asaba and 80% of samples from Enugu were positive, indicating that the milk samples may be soured and maybe unfit for processes that requires pasteurisation. These results fail the standard given by Thai Agricultural Standard

(TAS 6003-2010); there will be no protein precipitation under preliminary testing with 70% alcohol (TAS 6003-2010).

4.2.2.6 Titrable Acidity

The high percentage of acid present in milk samples at any time tells the age of the milk and possible contamination of the milk (Wanjala *et al*., 2017). The mean acidity was recorded as 0.689±0.07, 0.741±0.06, 0.744±0.06, 0.637±0.06 and 0.698±0.09 from Obinze, Owerri, Elele, Asaba and Enugu respectively. There was no significant difference ($P>0.05$) between the mean acidity of the samples from different locations. However, there was significant difference ($p<0.05$) between the mean acidity of samples from different locations and the declared standard value of ≤0.16% by Thai Agricultual Satandard (TAS 6003-2010). Wanjala *et al*. (2017) in his work suggested that high acidity may indicate high microbial and enzyme activity in the samples, which may be as a result of lack of adherence to the cold chain in the distribution channels and the long duration taken from the source to market. Wanjala *et al*. (2017) also reported that high acidity levels in raw milk could indicate high microbial load. This however, indicates why there was high total bacterial count in this study and reflects the substandard hygienic conditions during production and handling of milk in the area.

4.2.2.7 Alkaline Phosphotase activity

The mean p-nitrophenol concentrations were recorded as 0.418±0.025, 0.346±0.021, 0.408±0.020, 0.304±0.037 and 0.325±0.025 from Obinze, Owerri, Elele, Asaba, and Enugu respectively. There was no significant difference ($p>0.05$) between the mean p-nitophenol of the samples from different locations. However, there was significant difference ($p<0.05$) between the standard value of 0.01mg/ml published by Collins and Lyne (2004) and the mean

of samples from different locations. Although this test is best used for pasteurised milk sample (Collins & Lyne, 2004), using it for this unpasteurised *Nunu* samples also gives an information on possible microbial contamination because of high value of p-nitrophenol concentration indicates possible microbial contamination and inadequate pasteurisation for pasteurised milk samples. This however, conforms to the suggestion that these *Nunu* samples maybe contained during processing.

4.2.2.8 Heavy Metal Concentrations

Lead is one of the limited classes of element that can be described as purely toxic. High level of lead is particularly of great concern. It induces reduced cognitive development and intellectual performance in children, increase blood pressure, and cardiovascular diseases in adults as well as liver and kidney dysfunctioning (Ogabiela *et al.,* 2011). The level of lead observed in this study, however is below the tolerance limit of 0.02mg/l as reported by Omola *et al*. (2019). Low level of lead was also reported by Sarembayeva *et al.* (2020) for fermented milk in Kazakhstan, and Tona *et al.,* (2013). This result is lower than the result observed by Omola *et al*. (2019) and Ogabiela *et al*. (2011) for fermented milk in Kano.

Cadmium and solution of its compounds are toxic, and chronic exposure can cause damage to lungs and eventually death. When accumulates in the kidney and liver, it causes kidney dysfuntioning and liver failure (Abdallah, 2011). The level of Cadmium recorded in this study was below permissible limit of 0.075mg/l as reported by Ogabiela *et al.* (2011). The values in this study are lower than the values reported by Ogabiela *et al.* (2011), and Abdallah (2011).

Zinc is a component of many enzymes and proteins, and is involved in gene regulation (Omola *et al.,* 2019). It is an important element but high exposure results in anaemia, leucopenia, gastrointestinal diseases and diarrhea (Ogabiela *et al.,* 2011; Omola *et al*., 2019). The levels of

zinc observed in this study are lower than values reported by Ogabiela *et al.* (2011) and higher than the values reported by Omola *et al*. (2019).

4.2.3 Proximate Composition of *Nunu* samples

The milk protein particularly Caseins contains an appropriate amino acids, which contributes in the formation of hormones and enzymes, which controls a variety of body functions such as growth and repair of the body cells as a high energy resource for the body (Omola *et al.*, 2019). The protein obtained in this study showed significant difference between the different locations ($p<0.05$). Tukey pairwise comparison showed that samples from Asb (3.33%) had the highest mean protein content, and the Elbk sample had the lowest mean protein content of 2.28% which was not significantly different from Obz and Amah mean protein content. The mean protein content obtained in this study is within permissible limit of 3% by FAO (2004). The results are similar to the result reported by Obi and Ikenebomeh (2007) for *Nunu* in Benin-city, while it was lower than the result reported by Omola *et al*. (2019) for *Nunu* sold in Kano.

Carbohydrate is considered as fuel for all organisms, which contributes to about 55-75% of energy required by the organisms (WHO, 2004). The carbohydrate contents obtained in this study showed significant difference ($p<0.05$). The Tukey pairwise comparison showed that mean carbohydrate content from Amah (12.17%) and Obz (12.16%) was significantly higher than other locations, and samples from Asb had the least mean carbohydrate content (6.95%). The values from this study are higher than the values reported by Omola *et al.* (2019) for *Nunu* sold in Kano, and Uzeh *et al.* (2006).

The ash content accounts for the mineral constituents in the milk sample (Omola *et al.*, 2019). The ash is the inorganic residue remaining after the water and inorganic matter have been

removed by heating in the presence of oxidizing agent which provides a measure of the total amount of minerals present in a food (Omola *et al.,* 2019). In this study, ash content showed significant difference between the locations ($p<0.05$). The Tukey pairwise comparison showed that the mean ash content of Obz (1.32%) and Elbk (1.30%) was significantly higher than other locatons, with Asb samples having the lowest mean of (0.93%). The ash content in this study was higher than the report of Gemechu (2016) for whole Cow milk in Ethiopia. Also, it is higher than values reported by Omola *et al.* (2019) for *Nunu* sold in Kano. This result compared well with the result reported by Okeke *et al.* (2016). However, Tamime, (2009) revealed that the ash content of Cow milk ranged from 0.7-0.8% even though it is affected by feeding, breed, and stage of lactation.

The fat content of milk is of economic importance. It is a source of energy and contributes to the palatability of the diet (Omola *et al.,* 2019). Pieter *et al*. (2007) noted that milk is sold on the basis of its fat content but excess of it in food could be of health risk. The fat content in this study showed significant difference between the different locations ($p<0.05$). Tukey pairwise comparison showed that Enx sample (3.62%) had the highest mean fat content, and Obz samples had the lowest (3.40%). The fat content in this study showed similarity in the result reported by Omola *et al.,* (2019) in kano, and was lower than the result reported by Egwaikhide *et al.* (2014) in Kaduna. The values of the fat content from this study were within the FAO (2004) standard of 3.5%. The Low fat content of this study could be as a result of adequate churning done during processing of *Nunu.*

Moisture influences microbial growth. The tendency of microorganisms to grow in foods depends on the water content (Braide *et al.,* 2018). High moisture content is responsible for high bacterial growth in foods (Londhe, *et al.,* 2012) which decreases the shelf life of milk sample. Also low moisture content hinders bacterial growth (Londhe *et al.,* 2012). The moisture content in this study showed significant difference between the different locations ($p<0.05$).

The samples from Asb had the highest mean moisture content (85.12%), and sample from Amah had the least moisture content (80.76%) The moisture content from this study was comparable with the result of Omola *et al.* (2019) for *Nunu* sold in kano, but higher than the result reported by Noah and Salam (2020), the high moisture content observed in this study suggest the reason there was high bacterial content in this study.

Crude fibre was not detected in this study. This observation is similar to the report of Aliyu *et al.* (2019) for fermented milk in Nasarawa state, and that of Okiki *et al.* (2017).

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATION

5.1 Conclusion

From the findings of this study it is concluded that; *Nunu* produced by small scale producers in Enugu, Asaba, Elele, Owerri, and Obinze districts may be hazardous for human consumption and can be a potential source of milk-borne infections. Poor milking procedures, milk handling practices including the surrounding environment may contribute to the poor bacterial quality of the *Nunu*. This study revealed that *Nunu* samples analysed contained heavy metals such as cadmium, lead and zinc but within permissible level. The nutritional composition showed that the values for protein, fat, and carbohydrate were within permissible level.

However, consumption of *Nunu* in these five study areas at the time of this study may result into health problems. This is supported by evidence of pathogenic bacteria isolated in this study.

5.2 Recommendation

Based on the above conclusion, it is therefore recommended that; Routine assessment of *Nunu's* microbial quality produced by small-scale producers and consumed by public has to be mandatory in order to safeguard the public from milk-borne zoonotic infections which may radiate through consumption of unsafe milk and milk products.

There should be enlightenment programme by NGO's or the appropriate government agencies on good milking practices and good processing practices of *Nunu.* The production of *Nunu* for public consumption by individuals without appropriate permission by the appropriate authority should be discouraged. Studies on preservation methods of *Nunu* should be encouraged to maintain the nutritional quality of the *Nunu*.

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APPENDICES

ANOVA (Single factor) Result of total Bacterial count of 20 samples each of Five different locations.

Groups	*Count*	*Sum*	*Average*	*Variance*	*STD*	*SE*
Obz	20	193.38	9.669	0.065599	0.256123	0.057271
Amah	20	191.07	9.5535	0.064319	0.253611	0.056709
Elbk	20	191.63	9.5815	0.077908	0.27912	0.062413
Asb	20	191.48	9.574	0.086194	0.293588	0.065648
Enx	20	190.84	9.542	0.053343	0.230961	0.051645

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*

Between Groups	0.20051	4	0.0501275	0.721544	0.579315	2.467494
Within Groups	6.59989	95	0.069472526			
Total	6.8004	99				

ANOVA (Single factor) Result of Total *Campylobacter* count (log10 cfu/ml) of 20 samples each of Five different locations.

Groups	*Count*	*Sum*	*Average*	*Variance*	*STD*	*SE*
Obz	20	97.74	4.887	2.901348	1.703335	0.380877
Amah	20	69.9	3.495	6.969468	2.639975	0.590316
Elbk	20	88.81	4.4405	5.235584	2.28814	0.511644
Asb	20	77.23	3.8615	6.755856	2.599203	0.581199
Enx	20	69.73	3.4865	6.912982	2.629255	0.587919

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	30.25769	4	7.564424	1.314398	0.270236	2.467494
Within Groups	546.7295	95	5.755048			
Total	576.9872	99				

ANOVA (Single factor) Result of Average Total *Salmonella* count (log_{10} cfu/ml) of 20 samples each of Five different locations.

Groups	*Count*	*Sum*	*Average*	*Variance*	*STD*	*SE*

Obz	20	50.69	2.5345	4.527773	2.127857	0.475803
Amah	20	38.66	1.933	4.81918	2.195263	0.490876
Elbk	20	51.03	2.5515	4.587319	2.141803	0.478922
Asb	20	25.6	1.28	4.030442	2.007596	0.448912
Enx	20	37.39	1.8695	4.505752	2.122676	0.474645

Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	22.48167	4	5.620416	1.250623	0.294968	2.467494
Within Groups	426.9389	95	4.494093			
Total	449.4205	99				

ANOVA (Single factor) Result of Average Total Staphyloccocal count (log_{10} cfu/ml) of 20 samples each of Five different locations.

Groups	*Count*	*Sum*	*Average*	*Variance*	*STD*	*SE*
Obz	20	69.04	3.452	6.883101	2.623566	0.586647
Amah	20	69.36	3.468	6.870648	2.621192	0.586116
Elbk	20	100.87	5.0435	3.016087	1.736689	0.388335
Asb	20	67.2	3.36	7.95	2.819574	0.630476
Enx	20	104.04	5.202	1.55128	1.245504	0.278503

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*

Between Groups	69.42788	4	17.35697	3.303432	0.013995	2.467494
Within Groups	499.1512	95	5.254223			
Total	568.5791	99				

ANOVA (Single factor) Result of Average Total Coliform count (log_{10} cfu/ml) of 20 samples each of Five different locations.

Groups	*Count*	*Sum*	*Average*	*Variance*	*STD*	*SE*
Obz	20	83.81	4.1905	6.257384	2.501476	0.559347
Amah	20	61.63	3.0815	8.235656	2.869783	0.641703
Elbk	20	47.19	2.3595	7.199258	2.683143	0.599969
Asb	20	36.73	1.8365	8.285613	2.878474	0.643646
Enx	20	48.72	2.436	7.678857	2.771075	0.619631

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	65.31516	4	16.32879	2.168108	0.078438	2.467494
Within Groups	715.4786	95	7.531353			
Total	780.7937	99				

Absorbance of p-Nitrophenyl phosphate concentrations

CONCENTRATION (mg/ml)	ABSORBANCE @ 410nm
0.8	0.9
0.4	0.45
0.2	0.22
0.1	0.11

Anova (Single Factor) result for the Acidity values of 20 samples each of Five different locations.

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.153056	4	0.038264	1.070426	0.375554	2.467494
Within Groups	3.39592	95	0.035747			
Total	3.548976	99				

Anova (Single factor) result of Alkaline phosphate activity 20 samples each of Five different locations.

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.044696	4	0.011174	2.257376	0.06862	2.467494
Within Groups	0.470246	95	0.00495			
Total	0.514942	99				

Similarity in distribution of Bacteria in the locations

Location		Sorenson's Coefficient (CC)
Obz	Amah	0.89
Obz	Elbk	0.60
Obz	Enx	0.57
Obz	Asb	0.75
Amah	Elbk	0.80
Amah	Enx	0.71
Amah	Asb	0.88
Elbk	Enx	0.73
Elbk	Asb	0.92
Enx	Asb	0.67

ANOVA for Ash content of Nunu samples.

ANOVA

Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.594846	4	0.648712	174.5602	7.11E-4	2.467494

Within Groups	0.353045	95	0.003716
Total	2.947891	99	

Tukey Pairwise Comparisons For Ash

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
OBZ	20	1.3220	A
ELBK	20	1.3005	A
AMAH	20	1.2275	B
ENX	20	0.99400	C
ASB	20	0.93250	D

Means that do not share a letter are significantly different.

ANOVA for Carbohydrate of *Nunu* samples

ANOVA

Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	430.4449	4	107.6112	3885.605	2.5E-10	2.467494
Within Groups	2.63101	95	0.027695			
Total	433.0759	99				

Tukey Pairwise Comparisons For Carbohydrate

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
AMAH	20	12.1670	A
ELBK	20	12.1635	A
OBZ	20	11.0940	B
ENX	20	8.6340	C
ASB	20	6.9525	D

Means that do not share a letter are significantly different.

ANOVA for Protein of *Nunu* samples

ANOVA

Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	19.80551	4	4.951377	201.1489	1.83E-45	2.467494
Within Groups	2.33847	95	0.024615			
Total	22.14398	99				

Tukey Pairwise Comparisons For Protein

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
ASB	20	3.3355	A
ENX	20	3.0820	B
OBZ	20	2.3425	C
AMAH	20	2.3260	C
ELBK	20	2.2800	C

Means that do not share a letter are significantly different

ANOVA for Fat of *Nunu* samples

ANOVA

Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.52733	4	0.131833	43.46453	1.1E-20	2.467494
Within Groups	0.288145	95	0.003033			
Total	0.815475	99				

Tukey Pairwise Comparisons For Fat

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
ENX	20	3.6185	A
ASB	20	3.55300	B
AMAH	20	3.5205	B C
ELBK	20	3.48600	C
OBZ	20	3.3995	D

Means that do not share a letter are significantly different

ANOVA for moisture of *Nunu* samples

ANOVA

Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	296.2835	4	74.07086	973.93	3.69E-76	2.467494
Within Groups	7.22509	95	0.076054			
Total	303.5085	99				

Tukey Pairwise Comparisons for moisture

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
ASB	20	85.1225	A
ENX	20	83.6420	B
OBZ	20	81.7905	C
AMAH	20	80.7590	D
ELBK	20	80.7280	D

Means that do not share a letter are significantly different.

ANOVA for temperature of *Nunu* samples

ANOVA

Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.21985	4	0.554962	1.36815	0.250825	2.467494
Within Groups	38.53483	95	0.40563			
Total	40.75468	99				

Anova for Cd content of Nunu samples

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.98E-05	4	4.94E-06	0.536742	0.709063	2.467494
Within Groups	0.000874	95	9.2E-06			
Total	0.000894	99				

Anova for Pb content of Nunu samples

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	7.29E-05	4	1.82E-05	0.306452	0.873018	2.467494
Within Groups	0.005653	95	5.95E-05			
Total	0.005726	99				

Anova for Zn content of Nunu samples

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.3816	4	0.0954	3.475743	0.010753	2.467494
Within Groups	2.6075	95	0.027447			

Total	2.9891	99

Tukey Pairwise Comparisons for Zn

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping
ASB	20	0.8050	A
OBZ	20	0.7150	A B
AMAH	20	0.7050	A B
ELBK	20	0.6750	A B
ENX	20	0.6150	B

Means that do not share a letter are significantly different.