**CHAPTER ONE**

**1.0 INTRODUCTION**

Maize (Zea mays) is a cereal crop which is an important raw material in human diet (Amakoromo, 2011). It is an annual grass in the family Poaceae and is a staple food crop grown all over the world. It is believed that maize originated from Mexico and Central America. Fermented maize starch is also known as “pap”. It is also known as “Ogi” in the western part of Nigeria by the Yoruba’s or Akamu in the Eastern or “Akassa” in the North by the Igbo’s and Hausas respectively (Parveen and Hafiz, 2003). It is a fermented maize product obtained as smooth gel or mixed with boiling water to form a porridge, which has a sour taste. Similar maize preparations are referred to as “Akana” and “Kenkey” in Ghana (Parveen and Hafiz, 2003). It is a popular staple and most popular traditional weaning food in West African countries (Adams and Moss, 1995; Amakoromo, 2011). It is used as weaning food by low income earners who cannot afford the more expensive imported weaning foods. (Ozoh and Kuyanabana, 1995; Amakoromo, 2011).

“Ogi” is mostly prepared using traditional fermenting and malting technologies which are simple but do not guarantee quality and lack of contaminations as well as lack of the appropriate nutritive value (Marero *et al.,* 1989). It is prepared by soaking (steeping) in water for two to five days, grinding it (wet milling) and sieved to remove the husk. The main reason for fermenting maize grains is to convert starch contents in the cereals such that it does not require dilution. The fermenting process also removes the pathogens. Ogi provides about 20-26 kcal/kg per day to an infant who has an average density of 0.26 kcal/kg (Brown *et al.,* 1998).

In most parts of Africa especially in Nigeria, children are fed with mashed adult foods. These foods are bulky and can cause malnutrition. The developments of nutritionally balanced calorie as dense weaning foods lead to the fermentation of maize to provide pap. The food must also be of the right quantity to satisfy the infant at one feeding. It is also a choice of meal for patients in need of soft and easily digestible foods (Jay, 2005). They are important energy food rich in carbohydrate with traces of vitamins, proteins and minerals (Achtenberg *et al.,* 1994; FAO, 2009) and are natural antioxidants (Eaton and Nelson, 1991). Its reputation as the most popular traditional weaning food and its consumption by convalescent in the West African regions calls for a safe product, free of pathogen and any potentially hazardous microorganisms. The traditional fermentation processes of pap are usually spontaneous and uncontrolled (Odunfa, 1985) and have led to the loss of nutrients. The nutritive quality of maize porridge is very low resulting from low quality maize proteins and substantial loss of nutrients at the different stages of production (Nkama *et al.,* 2000).

The Microbiology of pap and its related products has been studied (Odunfa and Adeyele, 1985; Adegoke and Babbola, 1988; Hountunigha, 1994). New attention is presently on the use of starter cultures, which is solving numerous problems associated with the product capable of prevention and treatment of many water borne disease using bacteriogenic lactic acid bacteria (LAB) (Olukoya *et al.,* 1994). Olasupo *et al.* (1997) increased the shelf-life of “pap” using a bacteriocin producing *lactobacillus* isolate. “Ogi” is fairly acidic (pH 4.8), which tends to inhibit the growth of some bacteria. Despite the delicate health position of pap to some consumers, the role of spoilage microorganisms has not been investigated, nor has their potential to produce harmful metabolites. Its spoilage is however, enhanced by some extrinsic factors amongst which is storage. There are so many problems which can arise from fermentation of “Ogi” (i.e. spoilt ogi corn starch) and this may include; deriving complete sour taste which may result in over fermentation due to the conception of people. Also the length of fermentation can also affect the final product.

Pap as a fermented food contains bacteria and fungi as a result of the fermentation which takes place in the cereal starch (Odunfa 1985). Evaluation of the microbial quality of pap is to identify the contaminants associated with the improper storage for a relatively period of time. Improper storage is likely to develop other contaminants which can become harmful to consumers especially children; it could even lead to food poisoning or/and intoxication. Therefore, the study was carried out on the freshly prepared raw “Ogi” that was kept for seven days with one of the water been changed and the other not changed throughout the seven days.

**1.1 AIM**

The main of this study is to isolate and identify microorganisms especially bacteria and fungi from stored maize (Zea mays).

**1.2 SPECIFIC OBJECTIVES**

To isolate and identify bacterial contaminants from stored pap.

To isolate and identify fungal contaminants from stored pap.

**CHAPTER TWO**

**2.1 LITERATURE REVIEW**

Pap is fermented cereal porridge from West Africa, typically made from maize or millet (United Nations FAO, 2006). Traditionally, the grains are soaked in water for up to three days, before wet milling and sieving to remove husks. The filtered cereal is then allowed to ferment for up to three days until it sour. It is then boiled into pap, or cooked to make a stiff porridge. The fermentation of “Ogi” is performed by various lactic acid bacteria including *Lacto bacillus* Spp.and various yeasts including *Saccharomyces* and *Candida* Spp.

 Maize (Zea Mays L., Poaceae) is the most important cereal in the world after wheat and rice, with regard to cultivation areas and total production (Purseglove, 1992; Osagie and Eka, 1998). The name maize is derived from the South American Indian Arawak-Carib word “Mahiz”. It is also known as Indian corn or corn in America (Kochhar, 1986; Purseglove, 1992). It was introduced into Nigeria in the 16th Century by the Portuguese (Osagie and Eka, 1998). The global production of maize is estimated to about 300million tonnes per year. 145million (or about 50 percent) of which are produced in USA alone (Ihelarouye and Ngoddy, 1965; Kochhar, 1986, Purseglove, 1992). In Nigeria its production is quite common in all parts of the country, from the North to the South with an annual production of about 5.6million tones (Central Bank of Nigeria, 1992). The country’s maize crop covers about 1million hectares out of nine million hectares it occupies in Africa (Hartmans, 1985).

 Maize is prepared and consumed in a multitude of ways which varies from region to region is from one ethnic group to another ethnic group. For instance, maize grains are prepared by boiling or roasting as paste (‘eko’), abado’, and ‘elekute’ in Nigeria and ‘kenke’ in Ghana, or as popcorn which is eaten all over West Africa. Traditional methods of preparations and uses of maize are restricted to definite localities or ethnic groups. This trend was also noted in the traditional preparation and uses of cassava (*Manihot esculenta crantz, Euphorbiaceae*) by Etejere and Bhal (1985).

**2.2 USES OF MAIZE**

**2.2.1 Human Food**

 Maize & cornmeal (ground dried maize) constitute a staple food in many regions of the world. Maize is central to Mexican food. Virtually every dish in Mexican cuisine uses maize. One form of grain or cornmeal, maize is the main ingredient of tortillas, tamales, pozole, atole and all the dishes based on them, like tacos, quesadillas, chilaquiles, enchiladas, tostadas and many more. In Mexico even a fungus of maize known as *Huitlacoche* is considered a delicacy. Maize which is a major source of starch (Solon Robinson, 1853) is a major ingredient in home cooking and in many industrialized food products. Maize is also a major source of cooking oil (corn oil), and of maize gluten. Maize starch can also be hydrolyzed and enzymatically treated to produce syrups, particularly high fructose corn syrup, and sweetener; and also fermented and distilled to produce grain alcohol. Sometimes maize is used as the starch source of beer.

**2.2.2 Chemicals**

Starch from maize can also be made into plastics, fabrics, adhesives, and many other chemical products. The corn steep liquor, a plentiful watery by product of maize wet milling process, is widely used in the biochemical industry and research as a culture medium to grow many kinds of microorganisms (Liggett R. Winstons Koffler, H., 1948).

**2.2.3 Medicinal Uses**

 A crop which is highly edible and nutritious as maize, also has some medicinal uses among the local people. It is used to cure many diseases, which it had over the years proved to be very effective.

These include:

1. Water filtered through charcoal obtained from maize stalk can be used as a treatment to cure gonorrhea (Abdulrahman, 1997).
2. An infusion obtained from stigma of maize inflorescence can be used for treatment of diseases of the urinary tract or passage (Abdulrahman 1997).

Maize is consumed in many forms in different parts of the world, from maize grits, polenta and corn bread to popcorn and products such as maize flakes (Rooney and Serna Saldivar, 1987). The grain is fermented to give ogi in Nigeria (Oke, 1967) and other countries in Africa (Hesseltine, 1979) and is decorticated, degermed and precooked to be made into arepas in Colombia and Venezuela (Instituto de investigations Tecnologicas, 1971; Rodriguez, 1972).

Maize is also widely used to make beers in Benin for example; malt is obtained by germinating the grain for about five days. The malt is then exposed to the sun to stop germination. The grains are lightly crushed in a mortar or on a grinding stone. The malt is cooked and the extract is strained off, cooled and allowed to stand. After three days of fermentation it is ready to be drunk as beer, (FAO, 1981). The lime cooking process for maize is particular to Mexico and Central America (Bressani, 1990), although today technology has been exported to other countries such as the United States. A dough prepared from lime-cooked maize is the main ingredient for many popular dishes such as atole, a beverage with a great variety of flavors, and tamalitos, made by wrapping the dough in maize husks and steam-cooking it for 20 to 30minutes to gelatinize the starch. This form is usually prepared with young chipilin leaves (Crotalaria Longirostrata) the flowers of loroco (Fernaldia Pandurata) or cooked beans mixed with the dough thus improving the nutritional quality of the product and its flavour (Bressani, 1983). There are many other ways to convert maize into interesting and acceptable forms for human use.

**2.3 OGI MANUFACTURE/PAP MANUFACTURE**

Traditional process of making “ogi” has a number of slight variations described by several authors. “Ogi” is traditionally prepared in batches on a small scale of two or three times a week, depending on demand. The clean grain is steeped in water for one to four days to soften. Once soft, it is grounded with a grinding stone, pounded in a mortar or grounded with a power mill. The bran is sieved and washed away from the endosperm with plenty of water. Part of the germ is also separated in this operation. The filtrate is allowed to ferment for 24 to 72 hours to produce slurry which when boiled gives the ogi porridge. “Ogi” is usually marketed as a wet cake wrapped in leaves, or it may be diluted to 8 to 12percent solids in water and boiled into a pap or cooked to a stiff gel.

Akinrele (1970) reported that the souring of the maize took place spontaneously without the addition of inoculants or enzymes. He identified the organism involved in this unaided fermentation and investigated their effects on the nutritive value of the food. The moulds he identified are *Epholosporium, Fusarium, Aspergillus* and *Penicillium* species and the aerobic bacteria as *Corynebacterium* and *Aerobactor species*, while the main lactic acid bacterium he found was *Lactobacillus plantarum*. There were also yeast*: Candida mycoderma,* *Saccharomyce cerevisiae* and *Rhodotorula sp.* Although “ogi” is supposed to have an improved vitamin-B contents the result observed are quite variable, at least for thiamine, riboflavin and niacin. Banigo and Muller (1972) identified the carboxylic acids of pap fermentation. They found 11 acids, with lactic, acetic and butyric acids being the most important. The ogi making process is quite complex, and the porridge can also be prepared from sorghum, rice, millet and maize. Therefore, laboratory procedures have been developed to learn more about the process and introduce changes to convert the grains to food more efficiently. These have been described by (Akingbala, Rooney and Faubium (1981) and Akingbala *et al.,* ***(***1957). Whose studies have been useful in evaluating varieties of cereal grains for their efficiency in making “ogi” from whole male are kernels (79.1 percent) and dryed milled flour (79.8percent)

The commercial manufacture of ‘ogi’ does not differ substantially from the traditional method. Modifications have been introduced or added, such as the dry milling of maize into a fine meal or flour and subsequent inoculation of the flour-water mixture with a culture of *lactobacilli* and yeast. In view of the importance of ‘ogi’ in the Nigerian diet, large scale production is indicated. The material could be dried and packaged in polythene bags for a good shell life. This is why there is some problem in achieving a controlled fermentation with pure cultures. Some of the modification which include spray drying the slurry or drum drying.

**2.4 NUTRITIONAL AND SOME CHEMICAL CHANGES OF OGI AND OTHER FERMENTED MAIZE PRODUCTS**

**2.4.1 Chemical changes**

The process of fermenting maize, sorghum, or millet to produce pap not only removes parts of the maize kernels such as seed-coat and the germ, but also involves washing, sieving and decanting all of which induce changes in the chemical composition and nutritive value of the final product. Akinrele(1970) reported on specific nutrients of a number of ‘ogi’ samples produced in different ways unfermented and fermented with *Aerobactor cloacae, Lactobacillus* *plantarum* and a mixture of the two bacteria. He also compared the values found with those from the traditionally fermented products. Judging from the ratio of amino nitrogen to total nitrogen, the author reported that protein was degraded to a very small amount by any bacterial species. When compared with the unfermented ‘ogi’,*Aerobacter cloacae* appeared to synthesis more riboflavin and niacin ,which did not take place with *L.plantarum*. Traditionally produce ‘ogi’ had more thiamine and slightly lower the values of riboflavin and niacin than that made with maize and *A. cloacae.*

Akinrele (1970) and Banigo and Muller(1972) reported the carboxylic acids in ogi and found lactic acid in greatest concentration (0.55 percent) followed by acetic acid (0.99percent) and smaller amounts of butyric acid. The latter investigators suggested levels of 0.65 percent for the lactic acid and 0.11 percent for acetic acid, which are responsible for sour taste taste as goals for flavor evaluations. Banigo, de Man and Duitschaever (1974) reported on the proximate composition of Ogi made from common whole maize which were uncooked, and freeze- dried or cooked and freeze-dried after fermentation. Changes were relatively small in all major nutrients, with a slight increase in fiber and a decrease in ash content when compared with whole maize. These authors also reported on amino acid content, they found no differences maize flours and ‘ogi’ for all amino acids including the essential ones. The pap samples, however had about twice the amount of serine and somewhat higher values for glutamic acid. Adeniji and Potter (1978) reported that ogi processing did not decrease the protein content of maize, but total and available lysine were significantly reduced. On the other hand, tryptophan levels were more stable and in two samples increased, probably because of fermentation. These authors also found an increase in neutral detergent fibre and ash but no change in ligin. Akingbala *et al.* (1987) found a decrease in protein, either extract, ash and crude fiber in ogi as compared with maize that was processed as a whole grain or dry milled.

**2.4.2 Nutritional Changes**

The nutritional evaluations of Ogi and other maize fermented products are not readily available. Adeniji and Potter (1978) found a substantial decrease in protein quality of drum dried common maize ogi, which they ascribed to the drying process. These same authors reported significant losses on lysine. Several authors have more recently tested maize and sorghum and reported that fermentation improved the nutritional value of the product. Akinrele and Bassir (1967) found not protein utilization to those values in whole maize, even though some increase in thiamine and niacin was obtained. It has been indicated that some of the microorganisms responsible for ‘ogi; fermentation, such as *Enterobacter cloacae* and *Lactobacillus plantarum* use some of the amino acids for growth. This together with the elimination of the germ from Kernels explains that the very low protein quality of ogi and similarly produced maize products.

**2.4.3 Microbial Properties of Pap**

Three distinctive fermentative phases are characterized with pap production. At steepings gram negative organisms predominates especially *Achromobacter* and *Klebsiella spp*. Following the milling and sieving, gram negative organisms and lactic acid bacteria especially streptoccus spp dominates. The final stage of souring is dominated by non-homofermendative lactic bacteria especially *Lactobacillus plantarum* and *Pediococcus gunther* leads to the involvement of yeast as a minority component. *Saccharomyces cerevisiae* dominates the steeping stage while *Candida* survives in the finished product. Fermentation temperature is the major factor affecting the type of organisms involved in the production process. The lactic acid bacteria *Lactobacillio sp, Corynebacterium sp* and *Enterobacter sp* were among the major organisms responsible for the fermentation and nutritional improvement of pap (Akinrele 1970).

 At 150C, gram negative organisms survive in the products at the end of seven days; whereas at 330C and 370C, the flora is more heterogenous and the end product is accompanied by an odd odour and taste. Large fermentation rods suggestive of *Bacillus spp*. are also targeted to be the causal agent of proteolysis and the putrial colour of ‘ogi’. Also, the gas evolution that is found during steeping is attributed to the presence of *Klebsiella aerogenes*.

**2.4.4 Biochemistry of Pap**

When the grain is fermented, there is an increase in pH. The raw ‘ogi’ contains much less protein than the parent cereal because some soluble proteins are lost in steeping, washing with water and during mashing. Acid reacting substances are present during mashing. Acid reacting substances are also present during ogi fermentation and increases as the fermentation progresses.

Lactic acid is the primary volatile acid of ogi fermentation, acetic acid is the main volatile acid followed by butyric acids other volatile acids of fermentation are formic acid, propionic acid, isobutyric acid, isohexonic acid etc. during steeping of water it contains a higher amount of volatile acids unlike finished products, because the bulk of the acid produced in the later stages of the fermentation is leached out into the water. These acids appear in form of filing on the surface of the water.

**2.5 MICROBIAL CONTAMINATION AT DIFFERENT STAGES OF OGI PRODUCTION**

The outbreak of infectious and communicable diseases in tropical parts of the world is primarily as a result of food poisoning dur to microbial contamination (Jay, 2005). They are often responsible for acute gastroenteritis, abdominal discomfort and pain and diarrhea in infants and young adults (WHO, 2010; Kimmons *et al.,* 1999).

Maize grains were almost surfaced sterile prior to soaking. The isolated *Staphylococcus aureus* in few maize samples could have arisen from contaminated sacks used for storage and transportation of produce. Onovo and Ogaraku (2007) discovered some bacteria and fungi on exposed tigernet (*Cyperus* *esculentus* L.) before processing. The presence of *Aspergillus flavus, A. niger, Penicillium oxalicum, Fusarium oxysporium, Rhizopus stolonifer,* *Saccharomyces cerevisiae Candida albicans, Escherichia coli, Kebsiella aerogenes and Staphylococcus aureus* in water from the reservoirs suggests an extremely poor storage system deplorable sanitary conditions availab le at the three sites and multiple source of contamination due to open access to the reservoirs. Similar and related organisms were implicated in food and canned products by Gadaga *et al.,* (2008), Taulo *et al.,* (2009) and Oladipo and Omo-Adua (2011). Exposure of water to direct rays from the sun provided the required warmth and physical condition for growth and physical condition for growth of these organisms. Ozoh and Kuyanbana (1995) and Osho and Fagade (2000) equally verify water as the source of *Shigella spp* and *E. coli* in maize and other cereals porridge. Contaminated water was linked as the main source of *Vibrio cholera* infection (Shaheheraghi *et al.,* 2009) in some population in Iran. Oranusi *et al.* (2007) estimated 2-3 log10 coliforms per 100gml-1 of cooked maize porridge and linked contamination to the water used during the washing and soaking of maize grains. Heavy presence of *E. coli, Klesbsiella pneumonia* and *Streptococcus spp.* was reported in some food around the University of Ghana campus (Yeboah-Mu *et al.,* 2010). The introduction of *Salmonella sp., Rhizopus sp.,* and *Staphylococcus aureus* in some foods products have been linked to the presence of phytoxocin (Okafor & Omodamiro, 2006).

 The infinite and open access to the water tanks allowed cross contamination of the cooking utensils and bowls subsequent re-contamination of products at the later stages of production. The body swabs and underneath of nails contained substantial counts of *Staphylococcus aureus* and *Lactobacillus plantarum*, *E. coli, Klebsieela aerogenes and Saccharomyces cerevisiae*. The muslin clothes used in sieving the shaft were stained and soiled and often reused without thorough washing. The wrapping leaves and polythene bags were not sufficiently rinsed or sterilized before use. Omemu and Adeosun (2010) observed similar unhygienic practices among attendants and vendors at some production sites in Abeokuta, Nigeria. Air was laden with *Aspergillus flavus, Penicillium oxalicum* and *Rhizopus stolonifer* and served as a source of re-contamination of the finished products. Wacher *et al.* (1993) linked the contamination of freshly prepared pozol, traditional Mexican fermented maize dough to the surrounding air. The growth of bacteria (*Escherischia coli* and *Klebsiella aerogomes)* declined significantly in fully fermented wet paste as rightly observed by Byaruhanga *et al*.(1999) for *Bacillus cereus* after 24 hours fermentation. Also Mensah *et al.* (1990, 1991) observed a significant inhibition in the growth of some gram negative bacteria. Chukeatirote *et al.* (2010) observed an exponential increase in the population of bacteria and fungi with increased pH and fermentation time of grain.

 However, a re-contamination at latter stages of production by these enteric bacteria as observed could be linked to water as it was used repeatedly during preparation. Odugbemi *et al. (*1993) reported an increase in the level of faecal coliforms in cooked ogi under 9hours storage conditions and suggested a probable re-introduction during storage. A similar conclusion was held by Sanni *et al.,* (2002) for the rise in the population of yeast from 1.0 cfug-1 to 5.36 cfug-1 after 12 hours of fermentation. Alalade and Adeneye (2007) observed a significant correlation between pH and coliform bacterial count in wara cheese during fermentation process. Poor handling by vendors or sellers was rightly suggested by Wacher *et al.,* (1993) for the significant increase in enteric bacteria in freshly prepared pozol. On the other hand, the growth of *Lactobacillus plantarum* was unhindered at the different stages of production even after 48 hours of fermentation. Relatedly, an exponential increase in growth of some lactic acid bacteria was earlier reported by Kunene *et al.,* (1999) in both fermented and cooked maize porridge. During the preparation of pap, the critical points of contamination includes; the point of soaking the grains, milling and wrapping the products. Effective and good manufacturing practices (GMP) as recommended by Amoa-Awua *et al.,* (2007) would help eliminate contaminants for improved table quality and assure the health of consumers.

**CHAPTER THREE**

**3.0 MATERIALS AND METHODS**

**3.1 COLLECTION OF SAMPLE**

The raw pap samples were collected from Abakpa market Enugu in a sterile container and were transferred to the laboratory. (Microbiology laboratory of Godfrey Okoye University Ugwuomu-Nike Enugu) where the proper experiment was carried out.

**3.2 SAMPLE ANALYSES**

 The fresh raw pap was divided into two portions, sample 1 and sample 2. The samples were soaked in water for seven (7) days. The sample 1 was the pap that its water was not changed whereas sample 2 was the pap that its water was changed daily. The pH value of the steep water was checked throughout the experimental period.

**3.3. MEDIA PREPARATION**

 All the media used were prepared according to the manufacturer’s guide. Nutrient and MacConkey agar were used for the isolation of bacteria while Sabouraud dextrose agar (SDA) was used for the isolation of fungi.

**3.4 SERIAL DILUTION**

 Ten-fold serial dilution was carried out using sterile distilled water as the diluents. 10 test tubes containing ten milliliter volume per volume (10mllv/v) of sterile distilled water were used for each of the sample. They were labeled and arranged appropriately in a test tube rack. 10ml of the sample was serially diluted in test tubes containing 10ml of distilled water using a sterile syringe, each transfer was followed by a gentle agitation in other to mix the contents uniformly. The procedure was repeated for all the diluents in the same manner. The serial dilution was performed aseptically beside a lit Bunsen burner to prevent contamination.

**3.5 ISOLATION PROCEDURE**

 Each of the samples were isolated by inoculating respectively 0.1ml of 10^6 and 10^7 of the serially diluted pap samples in an already prepared Nutrient and MacConkey agar using the spread plate method. The sample was taken from the test tube aseptically using a sterile syringe near a lit Bunsen burner. The plate was allowed to gel properly and was incubated at 37C for 24-48 hours. This plate is known as the primary isolate. Fungi in the samples were isolated by inoculating 0.1ml of dilution 10^6 and 10^7 of the serially diluted samples in an already prepared petridish of Sabouraud dextrose agar using the pour plate technique. The plates were incubated at 25C for 3-5 days. This culture plate is also known as the primary isolate.

**3.6 PLATE COUNT**

 The Nutrient and MacConkey agar culture plate were examined after 24 hours of incubation. The number of bacteria colonies on the plate which had between 30-300 colonies were counted and the viable number /colony forming units was calculated while the colonies that were too numerous to count (TNTC) was discarded. The number of fungi colonies on the SDA plate was also calculated. The formula used to calculate is:

 Viable count/cfu/ml=Number of colonies formed on the plate / Volume of sample x Dilution factor

**3.7 ISOLATION OF PURE CULTURE**

 After incubation, the plates were examined for colonies that appeared different in their cultural characteristics. These colonies were collected with a sterile wire loop and was streaked on an already prepared Nutrient, MacConkey and SDA agar to obtain pure cultures. Each pure culture was then sub-cultured into agar slants in bijou bottles and kept as stock culture.

**3.8 IDENTIFICATION OF MICROBIAL ISOLATES**

 The identification of bacteria was carried out based on the classification scheme given in Bergey`s manual of determinative bacteriology. Bergey`s manual relied on the empirical classification system that separated all bacteria into 19 groups based on the basis of morphology, physiology, growth requirement and biochemistry. The discrete colonies of the bacteria isolates were identified based on the colony morphology, gram staining and biochemical tests. The fungi colonies were identified based on their daily cultural colony characteristics on SDA and microscopic characteristics after performing a slide culture technique and use of fungi atlas.

**3.8.1 GRAM STAINING**

* Place slide with heat fixed smear on staining tray.
* Gently flood smear with crystal violet and let stand for 1 minute.
* Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle.
* Gently flood the smear with gram’s iodine and let stand for 1 minute.
* Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle. The smear will appear as a purple circle on the slide.
* Decolourize using 95% ethyl alcohol or acetone. Tilt the slide slightly and apply the alcohol drop by drop for 5-10 seconds until the alcohol runs almost clear. Be careful not to over- decolourize.
* Immediately rinse with water.
* Gently flood with safranin to counter-stain and let stand for 45 seconds.
* Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle.
* Blot dry the slide with paper.
* View the smear using light microscope under oil immersion.

**3.8.2 BIOCHEMICAL TESTS**

CATALASE TEST

* Transfer a small amount of bacterial colony to a surface of clean dry glass slide using a loop or sterile wood stick.
* Place a drop of 3% H2O2 on to the slide and mix.
* Positive: Evolution of oxygen (within 5-10 seconds) as evidenced by bubbling.
* Negative: No bubbles or a few scattered bubbles.

COAGULASE

* Place a drop of normal saline on each end of a slide or on two or on two separate slides.
* With the loop, emulsify a portion of the isolated colony in each drop to make two thick suspensions.
* Add a drop of human or rabbit plasma to one of the suspensions and mix gently.
* Look for clumping of the organisms within 10 seconds.
* No plasma is added is to the second suspension to differentiate any granular appearance of the organism from true coagulase clumping.

CITRATE TEST

* Inoculate Simmons citrate agar lightly on the slant by touching the top of a needle to a colony that is 18 to 24 hours old.
* Incubate at 350c to 370c for 18 to 24 hours. Some organisms may require up to 7 days of incubation due to their limited rate of growth on citrate medium.
* Observe the development of blue colour denoting alkalization.
* Positive: colour change (prussian blue).
* Negative: no colour change.

INDOLE TEST

* Take a sterilized test tubes containing 4ml of tryptophan broth.
* Inoculate the tube aseptically by taking the growth from 18 to 24 hours culture.
* Incubate the tube at 370c for 24 to 28 hours.
* Add 0.5 ml of kovac’s reagent to the broth culture.
* Observe for the presence or absence of ring.
* Positive: Formation of pink or red colour (cherry-red ring).
* Negative: No colour change.

VOGES PROSKAEUR TEST

* Inoculate the test organism into the VP medium
* Incubate aerobically at 370c for 24 hours.
* Following 24 hour of incubation, aliquot 2ml of the broth to a clean test tube.
* Re-incubate the remaining broth for an addittional 24 hours.
* Add 6 drops of 5% alpha naphtol and mix well to aerate.
* Add 2 drops of 40% KOH, and mix well to aerate.
* Postive: Pink-red colouration surface within 30minutes (shake the tube vigorously during the 30 minute period).
* Negative: No colour change.

METHYL-RED TEST

* Inoculate two test tubes containing VP-MR broth with a pure culture of the organism under investigation.
* Incubate at 35oc for 4 days.
* Add 5 drops of MR indicate solution to the first tube (for VP test Barrit’s reagent to another tube).
* Positive: Red coloration.
* Negative: No colour change.

MOTILITY TEST: A clean grease free slide was labeled and 15-20ul of the culture was placed in the middle of the slide. A clean cover slip was lowered over the drop as though it were hinged at one side avoiding bubbles. The preparation was examined under the microscope first with x4 followed by x40 and x100 magnification. Then motile organisms will be identified. True bacterial motility must be differentiated from non-motile particles, which may show Brownian movement.

SUGAR FERMENTATION TEST

* Peptone water was prepared and distributed into labeled test tubes
* Seven (7) drops of Phenol red solution was added
* Glucose, Lactose, Sucrose, Maltose and Galactose (Carbohydrate source) were added to the peptone water contained in the labeled test tubes
* The prepared test media was sterilized at 121oC for 15 minutes
* The test samples were inoculated into the medium after the sample had cooled
* Sterile Durham tubes were inverted and placed into the tubes
* The test tubes were covered and incubated at 37oC for 18-24 hours
* Change in the colour of the medium from orange/red to yellow (indicated by the change in the colour of the Phenol indicator) indicates a positive result for acid production. No change in the medium colour indicates negative for acid production. Bubbles seen in the inverted Durham tubes indicate a positive result for gas production. No Bubble indicates a negative result.

**3.9** SLIDE CULTURE**:** Aseptically, with a pair of forceps, a sheet of sterile filter paper was placed in a petri dish. A sterile U-shaped glass rod was placed on the filter paper. About 4ml of sterile water was poured on the filter paper to completely moisten it. A sterile slide was placed on the U-shaped rod with the aid of forceps. A scalpel was flamed gently and used to cut 5mm square block of the medium from the plate of sabouraud’s agar and also transferred aseptically to the center of the slide. The four sides of the agar square were inoculated with spores or mycelia fragments of the fungi to be examined. Aseptically, a cover slip was placed on the surface of the agar cube and the petri dish was covered and incubated at room temperature for 48hours. After 48hours, the slides were examined for growth of hyphae and production of spores.

The slides were stained by placing a drop of lacto phenol cotton blue stain on a clean microscopic slide. The cover slip was removed from the slide culture and a drop of ethanol was added to the hyphae on the cover slip. As most of the ethanol evaporates, the cover glass, was placed mold side down on the drop of the lacto phenol cotton blue stain on the slide. It was then examined under microscope with x10 and x40 objective lens for its microscopic features.

**CHAPTER FOUR**

**4.1 RESULT**

The pH at the initial time of steeping the pap during storage at room temperature for seven (7) days was 6.5 and at the end of the steeping, the pH value reduced to 4.0. Table 1 shows the pH value during the steeping of pap. The decrease in pH may be as a result of the activities of Lactic acidbacteria which is responsible for the production of lactic acid during the steeping of the pap (Odunfa and Adeyele, 2000).

**Table 1: Change of pH Value during the Steeping of Pap for seven (7) days**

|  |  |  |  |
| --- | --- | --- | --- |
| Steeping time (days) | pH change |  |  |
| Day 1 | 6.5 |  |  |
| Day 2 | 6.0 |  |  |
| Day 3 | 6.0 |  |  |
| Day 4 | 6.1 |  |  |
| Day 5 | 5.0 |  |  |
| Day 6 | 4.7 |  |  |
| Day 7 | 4.0 |  |  |

**Table 2: Bacterial Counts of the Samples (Total Viable Counts in CFUml¯1)**

|  |  |  |  |
| --- | --- | --- | --- |
| S/N | Sample 1 (pap) |  | Sample 2 (pap) |
| 1 | 113 | 1.13 x 109 |  | 73 | 7.3 x 108 |
| 2 | 103 | 1.03 x 109 |  | 72 | 7.2 x 108 |
| 3 | 64 | 6.4 x 108 |  | 42 | 4.2 x 108 |

The study carried out on the Pap samples showed that Pap 1 had a higher total viable count for bacteria and fungi than Pap 2. The bacterial count of Pap 1 ranged from 1.13 x 109 to 6.4 x 108 cfu/ml, and 7.3 x 108 to 4.2 x 108 cfu/ml for Pap 2.

**Table 3: Fungal Counts of pap Samples (Total Viable Counts)**

|  |  |  |
| --- | --- | --- |
| Sample 1 (pap) |  | Sample 2 (pap) |
| 53 | 5.3 x 108 |  | 15 | 1.5 x 108 |
| 21 | 2.1 x 108 |  | 12 | 1.2 x 108 |

The fungal count for Pap 1 ranged from 5.3 x 108 to 2.1 x 108 cfu/ml and 1.5 x 108 to 1.2 x 108 for sample Pap 2.

**Table 4: Morphological and Biochemical Characteristics of Bacteria Isolates from Pap Samples**

|  |  |  |  |
| --- | --- | --- | --- |
| Isolates  | Biochemical test | Gram reaction, shape and arrangement  | Morphological characteristics  |
|  | CA | CO | IND | MR | VP | CI | OX | MOT | L | GL | S | MA | GAL |  |  |
| A | + | + | - | + | + | - | - | - | +/A | +/A | +/AG | + | + | Gram positive, cocci, clustered | Yellow colour |
| B | + |  | - |  |  | + |  | + | + | - | + | + | + | Gram positive, bacilli, rod, scattered | Long lods occurring in short chains |
| C | + | - | + | + | - | - | - | + | +/AG | +/AG | + | + | + | Gram negative, bacilli (rods) | Pink (short rod in clusters)  |
| D | + | - | - | - | - | + | + | + | + | - | - | + | + | Gram negative bacilli (rods) | Straight or slightly curved rod but not helical |

Probable organisms:

Isolate A *Staphylococcus aureus*

Isolate B *Lactobacillus plantarum*

Isolate C *Escherichia coli*

Isolate D *Pseudomonas aueroginosa*

Keys: CA= Catalase

CO= Coagulase

IND=Indole

MR=Methyl red

VP= Voges Proskauer

CL= Citrate utilization test

OX = Oxidase

MOT=Motility

L = Lactose

S = Sucrose

GAL= Galactose

GL= Glucose

MA= Maltose.

**Table 5: Colonial and Morphological Features of the Fungi Isolates from pap Samples**

|  |  |  |  |
| --- | --- | --- | --- |
| Isolates | Colonial features on Sabourand Dextose Agar | Morphological features on Microscope | Probable Organism  |
| E | The pigmentation was brown with milky centre. Colour of the underside was yellowish. The texture was globose to subglobose (powdery). The brown colour intensified and expands with age as the milky centre disappear  | Conidial heads are typically columnar and uniseriate with the phialides limited to the upper two thirds of the vesicle and curving to be roughly parallel to each other. | *Aspergillus* species  |
| F | Dark brown colonies with white light yellow and white periphery. Colour of the underside is black with light yellow edges | Conidial heads are dark brown to black, radiate and biseriate with metulae twice as long as the phialides. Conidial are globose, brown and rough walled | *Aspergillus* species |
| G | White colonies were formed which became light yellow to grey and to blackish grey with age. Light yellow under side was observed with fluffly texture | Non septate mycelia, sporangiospores are void in shape and are directly opposite to branched rhizoids | *Rhizopus* species |
| H | Cream white colonies which are flat, smooth, moist and glistening  | Blastoconidia are observed. They are unicellular, globose and ellipsoid to elongate in shape | *Saccharomyces* species |

**CHAPTER FIVE**

**5.1 DISCUSSION**

Bacteria and fungi are the most common contaminants of our fermented foods such as “pap”. They could easily contaminate the pap through spores of bacteria and fungi from the air. The presence of *Staphylococcus* Spp. and *Aspergillus* Spp. which are pathogenic may be able to produce toxins in the pap which are harmful to human especially infants and patients that needs easily digestible food. The pH change at the time of steeping the pap during storage at room temperature for the seven (7) days was 6.5 and later reduced to 4.0. The decrease in the pH change may be as a result of lactic acid bacteria which is responsible for the production of lactic acid during the steeping of pap (Odunfa and Adeyele, 2000). Bacteria isolated during the steeping of pap from day 1 is *Staphylococcus* Spp*.* while in day 2 *Staphylococcus* Spp.and *Escherichia coli* were isolated. Throughout the steeping of the pap from day 3 to day 7 *Staphylococcus* species*, Escherichia coli, Lactobacillus* species *and Pseudomonas* species became predominant. The fungi isolated throughout the steeping of pap from day 1 to day 7 were *Aspergillus* Spp., *Rhizopus* Spp., and *Saccharomyces* Spp. A total of eight (8) organisms were isolated and identified from the pap sample. The probable organisms isolated and identified includes; *Staphylococcus* specie, *Lactobacillus* specie, *Escherichia* *coli,* *Pseudomonas* specie, *Aspergillus* specie, *Rhizopus* specie, and *Saccharomyces* species. The bacterial and fungal counts recorded for both samples were found to be higher in pap sample 1 than in pap sample 2. The higher microbial load in Pap 1 may be due to buildup of micro-organisms in the water in which the pap was immersed and retained throughout the experimental period. The higher microbial load in pap 1 may also be as a result of the organism that is dominant in the pap during storage which are responsible for the fermentation of the pap and also as a result of the organism that contaminate the freshly prepared raw pap. The high viable count of organism in the fresh pap sample is also as a result of the abundant availability of nutrients in the fresh pap samples for the growth of microorganisms. The predominant organism in the pap are the *Lactobacillus* species. These organisms were responsible for the fermentation of the maize to form pap and ability to ferment certain carbohydrates (Odunfa, 1985; Amusa *et al.,* 2005; Ozoh Kuyanbana, 1995). Other organism dominant in the pap is the yeasts which include *Saccharomyces* *cerevisiae* which is also responsible for the fermentation of the pap. The presence of the *Lactobacillus* species and yeasts to the nutritional improvement of the ogi. This statement agrees with the findings of Akinrele (1970) as stated in the literature review. The presence of *Escherichia coli*, *Staphylococcus* *sp* and *Pseudomonas sp* in the sample could be as a result of contamination through handling and processing (Amusa et al., 2005). Fungi identified were *Aspergillus* Spp., *Rhizopus* Spp. and *Saccharomyces sp*. These microorganisms were in line with the identification by Akinrele (1970) and Odunfa, (1985). *Aspergillus* Spp. and *Saccharomyces* Spp. were also said be responsible for the fermentation and nutritional improvement of pap. *Aspergillus* Spp. and *Rhizopus* Spp. produce organic acids, while *Saccharomyces* Spp. contributed to the flavour development (Banigo and Muller, 1972).

**5.2 CONCLUSION**

From the results obtained in this work, *Staphylococcus* species and *Aspergillus* species are the most pathogenic organisms because of its ability to produce toxins. Pap can be kept at room temperature and the supernatant water changed daily. The presence of most of the microorganisms did not really show that the Pap was spoilt or of low quality, since most of the organism’s present were organisms associated with its production. It is undesirable, however, to have foods with high microbial load.

In conclusion, the research has shown that poor hygiene of sellers and environmental factors could cause the microbial contamination of pap sold in Abakpa market Enugu. Therefore, effective good manufacturing practices should be employed which may help eliminate the microbial contaminants for improved table quality and assure the health of consumers especially infant.