INTERNATIONAL JOURNAL OF PROGRESSIVE RESEARCH IN ENGINEERING MANAGEMENT AND SCIENCE (IJPREMS)

e-ISSN : 2583-1062

Impact Factor : 5.725

www.ijprems.com
editor@ijprems.com

Vol. 03, Issue 09, September 2023, pp : 409-415

IDENTIFICATION OF POTENTIAL MICROBIAL CONTAMINANTS FROM STORED PAP

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DOI: https://www.doi.org/10.58257/IJPREMS32060

ABSTRACT

Pap (ogi) is a common weaning food in West Africa. It is usually produced from the fermentation of maize or other cereals by the traditional fermentation method. Pap samples were collected from two different Market in Awka, Anambra State. This study isolated, identified and determined the bacterial and fungal isolated from pap in some selected market in Awka metropolis, Nigeria. The Organisms were isolated using the spread plate method and identification of resulting organisms on the basis of standard cultural, morphological and biochemical characteristics. The bacterial count recorded ranged from $9.5x10^5$ to $5.5x10^6$ CFU/ml while the fungal count ranged from $6.3x10^5$ to $3.6x10^6$ CFU/ml. Different bacterial and fungal species were isolated from the pap sample. The bacteria isolated include *Lactobacillus species*, *Streptococcus species* and *Stapyhlococccus species.* The isolated fungi genera include; *Aspergillus species*, and *Saccharomyces species*. This study has shown that poor hygiene of sellers and environmental factors could cause the microbial contamination of pap sold in markets. 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.

Keywords: *Ogi*, Microbial load, Eke Awka, Bacteria.

1. INTRODUCTION

Nigeria is endowed with a wide range of fermentable indigenous staple foods that serve as raw materials for agro-allied cottage industries. These industries utilize small-scale equipment and provide alternative equipment for rural communities while adding value to such local produce (Latunde-Dada, 2020). One common example of indigenous fermented foods in Nigeria is "Pap" (Fermented maize). 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. 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 (Francis, 2020). Microorganisms associated with the fermentation include bacteria, yeast and occasionally moulds (Anaukwu *et al.,* 2015). Pap (Akamu) is a product of fermented maize (*Zea mays*) widely eaten in Africa. 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. It is a popular staple and most popular traditional weaning food in West African countries (Amakoromo, 2011). It is used as weaning food by low income earners who cannot afford the more expensive imported weaning foods. (Ozoh, 2015). Pap meal is served in Nigeria as a weaning food for infants (1-3 year-old). Pap is widely used as the first native food given to babies at weaning to supplement breast milk and a major morning breakfast food for children and adults. Most preparation of pap meal is from cereals, namely, maize, guinea corn or millet readily available in all parts of the country (Onyekwere, *et al*., 2019). These cereals have similar chemical compositions of carbohydrates (68-88%), protein (9-15%), fat (3-5%) and vitamin B, range 0.45-0.6mg/100g. 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 (Akinrele, 2017). They are important energy food rich in carbohydrate with traces of vitamins, proteins and minerals and are natural antioxidants. The traditional fermentation processes of pap are usually spontaneous and uncontrolled (Odunfa, 2015) and have led to the loss of nutrients. "Pap" 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.,* 2019). 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. Pap provides about 20-26 kcal/kg per day to an infant who has an average density of 0.26 kcal/kg (Abdulmumeen, *et al*., 2012). Minimal processing technologies are non-thermal food processing technologies, which means the end product is comparable to the original raw material in terms

of freshness, naturalness, and nutritional content (Agu et al., 2014; Anaukwu *et al.,* 2015). The Microbiology of pap and its related products has been studied (Odunfa 2015). 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) (Galvez, *et al.,* 2017) increased the shelf-life of "pap" using a bacteriocin producing *lactobacillus* isolate. "Pap" 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 "Pap" (i.e. spoilt pap corn starch) and this may include; deriving complete sour taste which may result in over fermentation due to the conception of people (Teniola & Odunfa 2011). Also the length of fermentation can also affect the final product. Effective parameters useful in monitoring spoilage are also necessary in order to determine the appropriate time a fermentation should be terminated to avoid spoilage and harmful metabolite production (Teniola, 2011). Various microorganisms have been associated with the fermentation of pap (Ogi) as described by (Akinerele, 2017). They include *Cephalosporium, Aspergillus, Penicillium, Corynebacterium spp, Aerobactercloacee* and *Lactobacillus plantarum* among others. Pap as a fermented food contains bacteria and fungi as a result of the fermentation which takes place in the cereal starch (Odunfa 2015).

Fermented cereals like ogi, burukutu, fura, kunu, etc. are particularly important as weaning foods for infants and as dietary staples for adults (Mbachu *et al.,* 2014; Agu *et al.,* 2014; Awah *et al.,* 2016). The short shelf-life of these beverages are however a major problem faced by their producers and consumers. 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. The fermentation process of staples serves as a means of providing a major source of nourishment for large rural populations and contributes significantly to food security by increasing the range of raw material which can be used in the production of edible products. Fermentation enhances the nutrient content of foods through the biosynthesis of vitamins, essential amino acids and protein by improving protein quality and fibre digestibility. It also enhances micronutrients bioavailability and acids in degrading anti nutrient factors (Gabriel and Akharaiyi, 2017). The use of biological and natural means in the improvement of nutritive value of food products have greater advantages over the use of chemical because biotechnological synthesized products are less toxic and environmentally friendly (Motarjemi, 2012).

2. MATERIALS AND METHODS

Study Area

The study was conducted using a raw Pap (Akamu) sample purchased from Eke Awka Market in Awka, Anambra State.

Sample Collection

Pap (Akamu) samples were collected from Three (3) different markets location, Eke Awka, Agu Awka and Ifite. The Pap (Akamu) Samples were wrapped in a clean sterile polyethene bag to avoid further contamination and was taken to the Microbiology Laboratory for laboratory analysis.

Preparation of Culture Media

Media used: Media for the multiple tube fermentation and plate counts were prepared according to the manufacturer's instructions. The media used were Nutrient Agar (Himedia), and Sabouraud Dextrose Agar (SDA). All were prepared according to the manufacturer's instruction and sterilized in an autoclave at 121°C for 15 min.

Preparation of Blood Agar

5.6g of nutrient agar is measured and suspended in 200ml of distilled water. The mixture is heated to fully dissolve all components and then autoclaved at 121 degrees for 20mins. Once the nutrient agar has been autoclaved, it is left to cool but not to solidify, It is cooled t about 45 to 50 degrees then 5% (vol/vol) of sterile sheep blood is added and mixed gently but well. The mixture is then dispensed into sterile plates and allowed to solidify. Plates are then turned upside down and stored in refrigerator until they are ready to be used.

Materials used: Weighing balance, autoclave, Chloramphenicol, Nystatin, gas cylinder, measuring cylinder, spatula, beakers, cotton wool, 70% ethanol, distilled water, aluminium foil, conical flask, masking tape, Syringe, Petridish and lighter.

Microbiological Analysis Serial dilution of samples:

1g each of the Pap (akamu) sample was diluted in 9ml of sterile water. This was the stock sample and Ten-fold serial dilution was carried out. 1ml of diluent from each sample was transferred into 9ml of sterile distilled water, and then

aseptically, serial dilution was performed to obtain a suspension up to 10^-6. 1ml of each dilution (10^-5 and 10^-6) was inoculated on the Petri dishes using spread plate method. The plates were then incubated at 37°C for 18-24 hours.

Enumeration of Bacteria and Fungi:

Bacterial colonies that developed on culture plates were counted and expressed on Formula: CFU = Number of colonies x Dilution factor / Volume of sample

Pure Culture Maintenance:

The isolated organisms were purified through repeated subculture method. Streak plate methods were used for this purpose. Nutrient agar and Sabouraud Dextrose Agar (SDA) was used as media. When a plate yielded only one type of colony, the organisms were considered to be pure.

Identification and Characterization of Bacterial and Fungi Isolates.

Different morphological and biochemical characteristics accompanied with colony characteristics on different selective medium were observed for the identification of bacterial and fungi isolates.

Biochemical Tests.

Biochemical tests are used for microbial identification based on difference in their biochemical activities exhibited by different types of bacteria and fungi. Different biochemical tests are listed below that are used for identification of gram positive and negative organism.

Gram staining

A drop of normal saline was placed on a clean grease free slide, using a sterile wire loop, a smear of the culture was made on the slide and heat fixed. The fixed smear was covered with crystal violet stain for 60 second. The stain was rapidly washed off with clean water and drained quickly then covered with Lugols iodine for 60 seconds and was then washed off with clean water. The slide was flooded with 95% ethanol (decolorize) for 20 seconds. After which the slide was washed using distilled water and then flooded with safranin for 30seconds and then washed off. The back of the slide was then cleaned and placed in a draining rack for the stained smear to dry. The standard smear was then allowed to air dry and then viewed under the microscope using ×100 objectives lens with a drop of immersion oil.

Identification of Fungal Isolates

This was done based on the description of the gross morphological appearance of fungal colonies on the SDA culture medium and the modified slide culture technique using lactophenol cotton blue stain for the microscopic evaluation under X10 and X40 magnification of the microscope (Agu and Chidozie, 2021); with reference to the Manual of Fungal Atlases (Barnett and Hunter, 2018; Ellis *et al.,* 2017).

Citrate Utilization Test

The medium used was simmon's agar. The test is used to identify which of the organism can utilize citrate as the sole source of carbon for metabolism. Molten agar of citrate was dispensed into different tubes and allowed to cool. The test isolate was inoculated on the agar and incubated at 37ºC for 24 hours. At the end of 24 hours, the change in colour from green to blue indicated a positive result.

Catalase Test

Catalase test is performed to check the ability of bacterial isolates to produce the enzymes catalase that breaks Hydrogen Peroxide (H_2O_2) into water (H_2O) and Oxygen (O_2). A suspension of the organism was made on a clean grease free slide by emulsifying the organism with a loop full of normal saline. 3 drops of 3% hydrogen peroxide was added to the suspension and checked for the production of active bubbles which is a positive result.

Peptone Test

It is the simplest broth medium used for the growth of the organism and a base for determining carbohydrate fermentation patterns of non-fastidious micro-organisms. It is also used for the detection of indole production by the bacteria after the addition of Kovacs or Ehlrich reagent. Suspend 15.0 grams of the dehydrated medium in 1000 ml purified/distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Allow the medium to warm at 37°C or to room temperature before inoculating. Inoculate the specimen/ test organism. Incubate it aerobically at 35-37°C for 24 hours and examine for turbidity.

Sugar Fermentation Test

The sugars used in this test include sucrose, glucose, maltose and lactose. The media made of peptone (1.5g) n 100ml of distilled water and sugar 1g was dispensed into different test tubes containing inverted Durham tubes and bromothymol blue was added as he indicator , the test organisms was aseptically inoculated into the sugar solution, the medium mixed and incubated for 24hrs. The indicator and inverted Durham tubes were used to detect respectively the

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e-ISSN : 2583-1062

Impact Factor : 5.725

www.ijprems.com
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Vol. 03, Issue 09, September 2023, pp : 409-415

production of acid and liberation of gas by the isolates. The test tubes showing yellow colouration were recorded for acid production and Durham's tube showing gas bubbles were recorded as positive for gas production. It is a blood agar test. This test provides information on what hemolytic enzymes a bacterium possesses by providing a culture medium enriched with red blood cells, it is possible to determine whether a bacterium can destroy the cells and whether it can digest the hemoglobin inside. Hemolysis is determined by streaking for isolation on a blood agar plate, this might also include several stabs of the inoculum into the agar to encourage any anaerobic versions of the enzymes to digest blood cells. If the medium is discolored or darkened after growth, the organism has demonstrated alpha-hemolysis. If the medium has been cleared under growth, the organism is beta-hemolytic. No discernible change in the color of the medium constitutes gamma-hemolysis.

Motility Test

This test is used to determine whether an organism is motile or non-motile. Motile organisms contain flagella which helps them to travel beyond point of inoculation.

3. RESULTS

Table 1: Shows the results from the Spread plate counts. The total bacterial count ranges from 5.5 x 10^6 to 9.5 x 10^5 cfu/ml, where Sample 2 with the highest plate count which is 9.5x 10^5 cfu/ml and Sample 4 is 8.0 X 10^6cfu/ml.

Table 2: Shows the results from the plate counts. The total fungal count ranges from 6.3 x 10^5 to 3.6 x 10^6 cfu/ml, where Sample 5 with the highest plate count which is 6.3 x 10^5 cfu/ml and Sample 6 is 3.6 x 10^6cfu/ml.

Table 3: Shows the morphology/cultural characteristics of Bacterial Organism

Table 4: Shows the morphology/cultural characteristics of Fungal Organism

Table 5: Shows the Bacterial and Fungal isolates, the isolates were classified based on their gram stain reaction, shape under microscope and results yielded after some biochemical test was carried out on them.

Table 6: Shows the Sugar Fermentation of Bacterial and Fungal Isolates from Spoilt Pap

Table 7: Shows the Sugar Fermentation of Bacterial and Fungal Isolates from Fresh Pap

Table 1 The Bacterial Count of the isolates from Pap Sample

Pap Sample	Bacterial count (cfu/ml)
1	69 6.9 x 10^5
2	95 9.5 x 10^5
3	55 5.5 x 10^6
4	80 8.0 x 10^6

Table 2 Fungal Counts of pap Samples (Total Viable Counts)

Pap Sample	Fungal count (cfu/ml)
5	63 6.9 x 10^5
6	36 9.5 x 10^6

Table 3 Morphology Characterization of Bacterial Isolates

Isolates	Elevation	Margin	Colour	Opacity	Shape	Probable Isolate
1	Raised	Entire	Milky	Translucent	Circular	*Streptococcus Lactis*
2	Flat	Lobate	Creamy	Opaque	Irregular	*Lactobacillus acidophilus*
3	Raised	Entire	Yellowish	Translucent	Circular	*Staphylococcus aureus*
4	Flat	Lobate	Creamy	Opaque	Irregular	*Lactobacillus acidophilus*

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Table 4 Morphology Characterization of Fungal Isolates

Isolates	Elevation	Margin	Colour	Opacity	Shape	Probable Isolate
1	Raised	Entire	Milky	Opaque	Circular	*Aspergillus niger*
2	Raised	Entire	Creamy	Opaque	Circular	*Saccharomyces Cerevisiae*

Table 5 Biochemical Characterization of Bacterial isolate from Pap Sample

Isolates	Shape	Gram stain Reaction	CAT	IT	CUT	HT	MT	Organism Identified
Pap 1	Rod	+	+	-	-	-	+	*Streptococcus Lactis*
Pap 2	Rod	+	+	-	-	-	+	*Lactobacillus acidophilus*
Pap 3	Rod	+	+	-	-	-	+	*Staphyloccoccus aureus*
Pap 4	Rod	+	+	-	-	-	+	*Lactobacillus acidophilus*
Pap 5	Spherical	-	+	-	-	-	+	*Aspergillus niger*
Pap 6	Spherical	+	+	-	-	-	+	*Saccharomyces cerevisiae*

Key: + = Positive & - = Negative
CAT = Catalase test
IT = Indole test
MT = Motility test
CUT = Citrate utilization test
HT = Haemolysis test

Table 6 Sugar Fermentation of Bacterial and Fungal Isolates from Spoilt Pap

Isolates	Mannitol	Maltose	Lactose	Sucrose	Galatose
1	-	+	-	+	+
2	+	+	+	+	+
3	+	-	-	+	-
4	+	+	+	+	+
5	+	+	+	+	+
6	+	+	+	+	+

Table 7 Sugar Fermentation of Bacterial and Fungal Isolates from Fresh Pap

Isolates	Mannitol	Maltose	Lactose	Sucrose	Galatose
1	-	+	+	+	-
2	+	+	+	+	+
3	+	+	-	+	-
4	+	+	+	+	+
5	+	-	-	+	-
6	+	-	-	+	-

4. 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 probable organisms isolated and identified includes; *Staphylococcus* specie, *Lactobacillus* specie, *Streptoccocus* 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 2 than in pap sample 4 for the bacterial isolate count while pap sample 5 is higher than sample 6 for the fungal isolate count. The higher microbial load in Pap 2 and 4 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, 2015; Amusa *et al.*, 2015; Ozoh, 2015). 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 (pap). The presence of *Streptococcus sp*, and *Staphylococcus sp* in the sample could be as a result of contamination through handling and processing (Amusa *et al.*, 2015). Fungi identified were *Aspergillus* Spp., *Rhizopus* Spp. and *Saccharomyces sp*. These microorganisms were in line with the identification by (Akinrele 2017 & Odunfa, 2015). *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.

5. 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 Eke Awka market Awka, Anambra State. 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.

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