BACTERIOLOGICAL AND BIOCHEMICAL STUDY OF FISH FEEDS AND AQUACULTURE

BY

Precious OGIEMWONYI (Miss)

LSC1504782

DEPARTMENT OF MICROBIOLOGY,

FACULTY OF LIFE SCIENCES,

UNIVERSITY OF BENIN,

BENIN CITY.

JUNE, 2021.

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A PROJECT SUBMITTED TO THE DEPARTMENT OF MICROBIOLOGY, FACULTY OF LIFE SCIENCES, UNIVERSITY OF BENIN, BENIN CITY, EDO STATE, NIGERIA

IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF THE DEGREE OF B. Sc. (HONS) IN MICROBIOLOGY

JUNE, 2021.

CERTIFICATION

This is to certify that this project work was titled "**BACTERIOLOGICAL AND BIOCHEMICAL STUDY OF FISH FEEDS AND AQUACULTURE** was carried out by **PRECIOUS OGIEMWONYI** in the Department of Microbiology, Faculty of Life Sciences, University of Benin, Benin City, Edo state under my supervision.

_____ _____

MRS. F. O. OMOROTIONMWAN **Date**

(Project Supervisor)

APPROVAL

This project work is accepted in partial fulfillment for the award of Bachelor of Science, B. Sc (Hons.) in the Department of Microbiology, University of Benin, Benin City.

PROF. S. E. OMONIGHO **Date**

(Head of Department)

DEDICATION

I dedicate this project work to God Almighty and also to my loving parents Mr. and Mrs. C.A. Ogiemwonyi.

ACKOWLEDGMENTS

My profound gratitude goes to my project supervisor Mrs. F. O. Omorotionmwan, who was very supportive throughout the course of this study.

My sincere appreciation also goes to my lovely parents Mr. and Mrs. C.A. Ogiemwonyi and my siblings for their great care, support and love.

I also wish to say a very big thank you to Dr. A.G. Ogofure, my colleagues and also to my friends: Jennifer, Aisosa, Favour, Dominic, Esther, Ese, Tobin, Benjamin and Moses for their support.

My special gratitude goes to God Almighty for helping bring this project wok into completion.

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ABSTRACT

The bacteriological and biochemical quality of fish feeds used by fish farmers in Ikpoba-Okha and Egor local government area in metropolis were evaluated in this study. A total of twelve (12) samples were assessed from both locations. Questionnaires were administered to the farmers to get their informed consent. Samples were collected and analyzed in the laboratory within 1-5 hours after collection, standard microbiological analysis involving total heterotrophic count of the fish feeds, total coliform bacteria count of the fish feeds, Identification of the bacteria most prevalent in the feeds and antibiotics susceptibility pattern of the bacterial isolates. The results revealed that the mean value of total heterotrophic bacteria count for fish feeds obtained from Ikpoba-Okha was $1.72\pm7.02x10^2$ cfu/ml while Egor had a mean value of $5.20\pm2.13x10^2$ cfu/ml. The total coliform bacteria count for Ikpoba-Okha was $0.2\pm0.82x10^2$cfu/ml and Egor, $0.78\pm3.16x10^2$cfu/ml. The prevalence of bacteria isolates in fish feeds obtained from both Ikpoba-Okha and Egor local government areas revealed that *Escherichia coli* was most prevalent with a percentage value of 20.00% while *Actinobacter* was least prevalent with a percentage value of 2.50%. *Salmonella, Klebsiella* sp*, Citrobacter, Enterobacter aerogenes, Staphylococcus aureus, Micrococcus luteus, Chromobacterium, Proteus vulgaris and Shigella* sp. Were also present. Antibiotic susceptibility test revealed that all Gram positive bacteria identified in this study was susceptible to gentamycin and ofloxacin, meanwhile all bacteria identified in this study were resistant to ceftriaxone/ceftrazone.

CHAPTER ONE

1.1 INTRODUCTION

Like every other animal fish requires essential food nutrients to grow well, they must be able to find enough to eat at various stages of their lives. Natural feeds such as microscopic plants (phytoplankton) eg *Chlorella, Anabaena, Diatom cyclotella, Microcystis, Spirogyra, Ulotthrix* etc. and microscopic animals (zooplankton) are readily available for uncultivated fish as their primary source of food (Frigg, 1990). Due to high demands of healthy freshwater fish by man, fish is often removed from the wild to be cultivated. However, fish taken to be cultivated outside its natural habitat must be provided with adequate food supply (mostly based on artificial or prepared fish feed). Artificial fish feeds can either be complete or supplemental (incomplete diet). Complete diet, as the name implies supply all the ingredients ranging from carbohydrates, fats, vitamins and minerals, necessary for optimal growth and health of the fish. Meanwhile incomplete diet do not contain complete complement of minerals or vitamins. They are usually used to help support the naturally available diet with extra nutrients such as carbohydrate, protein and/or lipids (Gatlin *et al.,* 2002). Most fish farmers usually use the complete diet because of its high quality and nutritional value.

Generally, fish feeds may be contaminated because they are in constant contact with the environment, this therefore exposes them to microorganisms present in the environment. Specifically, feeds can be contaminated during processing, storage, transport or handling.

Environmental factors during storage predisposes fish feeds to microbial spoilage (FAO, 1998). Intake of contaminated feeds can cause diseases in fish. The presence of fungi in fish feeds can cause the disease aflatoxicosis (Russo *et al.,* 2010). Aflatoxicosis arises when feed contaminated with aflatoxins are consumed by fish (Ashley, 1970). Aflatoxins are chemicals produced by some species of fungi (*Aspergilus flavus* and *Aspergilus parasiticus*) also known as molds (Russo *et al.,* 2010). The disease can also affect man through the consumption of already contaminated fish. Aflatoxins causes immunosuppression, they are also potent carcinogens and may affect all organ systems, they cause liver cancer and have been linked to other types of cancer (Ashley, 1970). Also, pathogenic bacteria such as *Escherichia coli, Salmonella, Staphylococccus aureus, Proteus mirabilis* etc. have been seen to contaminate fish feeds (Ubiebi *et al.,* 2017).

The presence of these microorganisms in fish feeds depends on the storage conditions of the feeds, specifically temperature (Olayiwole *et al.,* 2015). Temperature and the level of hygiene during fish feed processing plays a key role in determining the level of risk of microbial contamination. Microorganisms can survive and easily proliferate in feeds stored at improper storage temperature.

1.2 AIM

This project study is aimed at analyzing the bacteriological and biochemical quality of fish feed in Benin City, Edo state.

1.3 SPECIFIC OBJECTIVES

The specific objectives were to:

1. Determine total heterotrophic plate count of fish feeds
2. Identify bacterial isolates
3. Determine total coliform bacteria count of fish feeds
4. Determine antibiotic sensitivity pattern of bacteria isolates

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1. FISH FEEDS FORMULATION

Feeding fish with just a single food type does not supply it with all the necessary nutrients, hence the need for diet formulation. Diet formulation is the process of blending feed ingredients with different vitamins and mineral supplements for the production of one diet with the required the required amount of essential nutrients (Hardy *et al.,* 2002). It is important that feeds produced for the culturing of fish are in good quality, the production of good quality fish is highly dependent on the quality of the ingredients used. A diet could either be formulated to serve as supplement or as a complete formulation i.e where only the complete formulation is used to feed fish without adding any other food. For fish to be fed with a complete diet without the addition of natural foods, then the complete feed must be nutritionally balanced, palatable, water stable and have proper size and texture, the feed may also be supplemented with natural or synthetic pigments (Chakraborty *et al.,* 2019).

Formulated feeds can either be dry, with a moisture content of 6-10%, semi-moist, with a moisture content of 35-40% or wet, with a moisture content of 50-70% (Chakraborty *et al.,* 2019). The process of extrusion and steam pelleting are commonly used for the production of dry feeds, diets formulated for warm water fish like catfish makes use of any of these processes, particularly extrusion. Extrusion is the key process in feed production, the physical product quality, pellet expansion and oil adsorption capacity are defined in this unit. It involves cooking of feed mix in the extruder barrel, by mechanical energy

dissipated into heat and the addition of water and/or steam. It is more expensive, requires higher temperature, pressure and moisture compared to the steam pelleting process, which only involves the addition of steam to the feed in a conditioning chamber where it is mixed with feed to raise moisture content and temperature of 15-18% and 70-80˚C respectively. Flakes and pellets are both types of dry feeds. Flakes consists of complex ingredients and pigments, they are made into a slurry, cooked and rolled over drums heated by steams. While pellets are made by extrusion or by steam, they can be made to either sink or float due to certain changes in diet composition and processing conditions. Semi-moist and wet feeds are made from single or mixed ingredients. They contain variable quantity of fish, crustacean waste, liver, slaughter-house byproducts, etc. and/or fish silage, dry ingredients like fish meal, whey, rice and wheat byproducts, vitamins and mineral supplements, and hydrocolloidal binding agents. Storing of wet feeds improperly or for a long period of time can affect its vitamin stability, oxidation of lipids and microbial contamination.

In order to prevent or slow down the growth of microorganisms, reduce the level of water activity and reduce loss of vitamin C, humectants are added to the fish diets (FAO, 1998).

2.1. NUTRITIONAL VALUE OF FEEDS

The quality of fish feeds plays a vital role in the health and quality of fish however, the nutritional content of the feed depends on what species of fish is being cultured and at what life stage. The commonly cultured species of fish are the tilapia and the catfish particularly the catfish. When fish are reared, their ability of being able to feed of natural food (algae, aquatic

plants, aquatic invertebrates etc.) becomes very slim or even impossible, they must be fed with a complete diet (proteins, carbohydrates, fats, vitamins and minerals) (Gatlin, 2002).

Proteins

They are formed by the linkage of individual amino acids, there are more 200 amino acids that occurs in nature and out of which only 20 of the 200 amino acids are common and out of these 20, 10 are essential amino acids but cannot be synthesized by fish. Methionine, arginine, threonine, tryptophan, histidine, isoleucine, lysine, leucine, valine and phenylalanine are the 10 essential amino acids that must be supplied by the diet.

Recently, fish meal has become the most expensive protein ingredient in aquaculture feeds. Increase use of plant proteins (eg soybean meal) can reduce the cost of fish meal. Soybean meal is frequently used as a fish meal replacer in diets because of its high protein content and quite a reasonable amount of amino acid, it also has a reasonable price and steady supply (Chou *et al.,* 2004). However, fish feeds prepared with plant proteins are typically low in methionine. Fish feeds can also be produced with yeast or bacterial, but fish produced using these microorganisms are usually deficient in methionine and lysine. Hence these deficiency must be supplemented to diets when these sources are used as dietary proteins and specific amino acids are very important requirements for optimal health and growth in fish.

As fish grows bigger, their protein requirements usually decreases. Hence protein requirements for bigger fish are low, while protein requirements for smaller fish are high.

Protein composition

Protein are composed of 50% carbon, 16% nitrogen, 21.5% oxygen, 6.5% hydrogen and 6% other elements. As much as 65% of proteins consumed by fish can be lost to the environment. Fish excretes nitrogen in form of ammonia (NH3) through their gills and then pass out 10% as solid waste.

Carbohydrates

Carbohydrates (starches, sugars and celluloses) are naturally occurring compounds consisting of molecules of carbon, hydrogen and oxygen. They are one of the most abundant organic substances in nature, formed by green plants from carbon dioxide and water during the process of photosynthesis. Carbohydrates are included in diets to help reduce cost of feeds and also because of their binding activity during feed production, they allow for the expansion of extruded feed pellets, making them water-stable and are able to float on surfaces of water (Hemre *et al*., 2002).

They can be divided into two fractions, the indigestible (3-7%) and the digestible fractions (35-40% or more for warm water fish). The ability to use up carbohydrates as a source of energy differs among fish species. Fresh water and warm water fish including catfish due to the fact that they have a much higher intestinal concentration of enzymes needed for the utilization of starch, are able to use up higher level of digestible carbohydrates better than cold water and marine water fish (Hemre *et al.,*2002). Feeds of warm water fish contains fairly large amount of grains or grain by-products that are rich in starch.

Lipids (fats and oils)

These are nutrients high and energy and can be used as a substitute for proteins in fish diets, as they have about twice the energy of both carbohydrates and proteins. Lipids are highly digestible, about 97% digestible by commonly cultured fish (catfish), they contain fatty acids and triacylglycerols. Lipids are not just a good source of energy, they are also a good source of essential fatty acids (EFA), they also act as a vehicle for absorption of fat-soluble vitamins and are precursors for steroid hormones and other compounds (Sargent, 1993). Essential fatty acids can be classified based on their chemical structures and can be either omega-3 (n-3) or omega-6 (n-6) fatty acids, which are both required by fish (depending on the specie). Fatty acids can either be saturated, polyunsaturated or highly unsaturated. Marine fish and algal are good naturally contains highly unsaturated omega-3 fatty acids and as such regarded as good sources of lipids incorporated into fish feeds during the process of production (Sargent, 1993).

Since lipids are high sources of energy and can spare protein, then it should be added to fish feeds. However, excess dietary lipids could cause problems such as too much fat deposition in the liver, thereby leading to decline in fish health, quality and storage of final product. Generally, the weight gain of catfish can be depressed when fed with feeds containing 15% or more lipids, it can also depress the feed's efficiency. Although this is not an issue because lipids level of commercial feeds for food-sized catfish barely exceeds 5-6%, about 3-4% are inherent in the feed ingredients while the remaining 1-2% are being sprayed onto the fish pellets.

Vitamins

Generally, vitamins are organic compounds necessary (in small amount) in the diet for the health, growth and reproduction of the fish. Vitamins are divided into two groups based on solubility. They are fat-soluble and water-soluble vitamins.

Fat-soluble vitamins include vitamin A (retinol, beta-carotene), vitamin D (cholecalciferol), vitamin E (tocopherol) and vitamin K (phylloquinone). Amongst these four, only vitamin E (tocopherol) acts as an antioxidant, it also inhibits dietary lipid oxidation, thereby improving shelf life (Vera, 1930).

Water-soluble vitamins include vitamin C (ascorbic acid), thiamin, riboflavin, niacin, vitamin B_6 (pyridoxine, pyridoxal and pyridoxamine), folacin, vitamin B_{12}, biotin and pantothenic acid. Most of these vitamins are components of coenzymes that have specific metabolic functions, with vitamin C being the most important because of it is a very active antioxidant which helps boost the immune system of fish (Sinha *et al.,* 1994). Vitamin deficiency is rarely seen in fish, the most common that can ever occur are reduction in growth.

Minerals

Minerals are chemical elements required in diet for normal body function. They can be divided into two groups- macrominerals (calcium, phosphorus, magnesium, sodium, potassium, chloride and sulphur) and microminerals (iron, manganese, copper, iodine, zinc, cobalt, fluoride and selenium). Macrominerals helps to regulate osmotic balance and also helps in bone formation while microminerals are required by the body, only in trace amounts (Glencross *et al.,* 2007) .

2.2. COMPOSITION OF FISH FEEDS

Commercial fish feeds are composed of a mixture of vitamins, mineral premixes and feedstuffs (Protein feedstuffs and energy feedstuffs)

Protein feedstuffs

Protein feedstuffs contains 20 percent protein or more, they can be further subdivided into animal protein feedstuffs and plant protein feedstuffs. The most important source of proteins used in cultured fish (mainly cat fish) feeds are oilseed meals, peanut meal and canola meals.

Animal protein feedstuffs includes*;*

1. **Fish meal**: Fish meal is a valuable animal protein supplement and a supply of vitamins and minerals, it is made by prepared from dried, ground tissues of undecomposed, whole marine fish or fish cuttings. Certain species such of oily fish, such as menhaden, anchovy, herring and pilchard are the main source of fish meal and fish oil. Fish meal provides a concentrated source of high quality protein and a fat rich in omega-3 fatty acid, docosahexaeonic acid (DHA) and eicosapentaenoic acid (EPA). Fish meal although expensive, still remains the major component/ingredient in the formulation of commercial fish feeds (Rumsey, 1993).

Protein: The protein contained in fishmeal is rich in essential amino acids in a highly digestible form, particularly methionine pluscistine, lysine, threonine and tryptophan.

Fat: With omega-3 fatty acids provided as docosahexaeonic acid (DHA) and eicosapentaenoic acid (EPA), general health of the animal is improved.

2. Meat and bone meal: Meat and bone meal contains good quality proteins as well as a rich source of calcium and phosphorous, it is the rendered product from porcine meat (pork) or beef processing, void of blood, hair, hoof, horn, hide trimmings, manure or stomach content except in amounts as may occur unavoidably in good processing practices. The protein contained in meat and bone meal is minimal compared to the entire fish meal because it contains less lysine. It is a good source of minerals, it also has a high ash content (the high ash content may limit its use because of possible mineral imbalance). Porcine meat and bone/blood meal blend is an excellent protein source for use in cultured fish, particularly catfish. It is recommended that for catfish, the level of meat and bone meal (blends containing porcine meal and blood) should be 15 percent of the diet.

3. Poultry By-Product Meal: Poultry by-product meals consists of ground rendered clean parts of the carcass of the slaughtered poultry such as necks, feet, undeveloped eggs and intestines, exclusive of feathers, except in the amounts as might occur unavoidably in good processing practices (Cruz-Suarez *et al.,* 2007). The product contains approximately 58-65%, it is also a very good product for use in catfish feeds but its use it limited because it is not available on a regular basis at a reasonable cost per unit protein

4. Fish Offal Meal: Fish offal consists of fish gut/intestine. It is of better nutritional quality than meat and bone meal, but not as good as fish meal. Catfish offal meals contains approximately 50 percent protein, it is highly palatable to catfish.

Plant protein feedstuffs

The major plant protein sources used in cultured fish, particularly catfish includes soyabean meal and cottonseed meal. Others include canola meal, peanut meal and sunflower meal.

1. Soybean meal: It is prepared by grinding the flakes after removal of the oil from dehulled soybeans by solvent extraction. Trypsin inhibitors are either destroyed or reduced due to the level of heat applied during the extraction process. Soybean meal has the best amino acid profile of all common plant protein sources and it is highly digestible to catfish. Soybean meal has been the most frequently studied dietary ingredient as a fish meal replacer in diets for many fish species because of its high protein content, relatively well balanced amino acid profiles, reasonable price and steady supply (Boonyaratpalin, 1998).

2. Cottonseed Meal: Solvent-extracted cottonseed meal is prepared by grinding the cake remaining after the oil has been extracted. Cotton meal has long been used in diets for both terrestrial animals and fish because of its high protein content, availability and low cost. Cottonseed meal contains gossypol, which is toxic to fish leading to a restriction of its use as a fish feed ingredient (Herman, 1970).

Energy feedstuffs

The amount of crude protein found in energy feedstuffs is less than 20%. Grain and grain by-products, including corn grain, corn gluten feed, corn germ meal, wheat grain, wheat middlings, rice bran and animal and plant fats are commonly used in commercial fish feeds.

2.3 SOURCES OF CONTAMINTION IN FISH FEEDS

Feeds have been shown to be a major vector for transmission of bacteria to farms (Petreska *et al.,* 2013). Disease causing agents can be easily transmitted from animals through contaminated feed, they can also be transmitted to man through the consumption of fish, as fish serves as major source of protein in man's diet. Fish and fish products, particularly raw or undercooked products, have been involved in outbreaks associated with bacterial pathogens, biotoxins, histamine, viruses and parasites (Crump *et al.,* 2003).

Commercial fish feeds can easily get contaminated due to a number of factors. Commercial feeds are in constant contact with environmental organism, as a result it becomes easy for these environmental organism to become habitual in them. Environmental factors during storage exposes fish feeds to microbial spoilage (FAO, 1998). Microorganisms which are biological contaminants of the natural environment and are present in all feedstuffs. Although processing of feed materials helps to eliminate most of the normal micro flora, but some microorganisms with highly resistant spores are not eliminated.

Additionally, fish feeds can get contaminated through already contaminated ingredients selected for feed production. Crops such as cottonseed and peanuts are high aflatoxin risks because *Aspergillus flavus* often infest on them as a practically pure culture with few or no other microflora. Maize and sorghum grown in the tropics also pose high risk. Sometimes, famers in rural settings have to travel long distances to urban cities in order to obtain feeds for their fish, incurring longer transport times under suboptimal conditions of heat and humidity, resulting in spoilage and microbial contamination. These contaminants are pathogenic to fish as well as humans. Fish feeds can serve as carrier for a range of microbial contaminants (Maciorowski *et al.,* 2007). Bacterial contamination of feed ingredients or diets with potential pathogens will compromise fish and human health.

Furthermore, packaging and packaging materials as well as handling circumstances, including the nature and extent of quality control measures greatly influence the source and degree of contamination

(Zmyslowska, 2000). Feeds also stands the risk of getting contaminated if not properly stored. Insects such as moth larvae, beetles and weevils feeds on most ingredients used in production of feeds and then contaminate them with faeces, webbing, body parts, foul odour and microorganisms. Intense insect activity often leads to mould growth, complete destruction of feeds and even pose serious health risks to fish feeding on the already damaged/contaminated ration.

Due to the incessant rise in cost and shortage in supply of fishmeal, which is one of the main ingredients for the production of commercial fish feeds, several companies has resulted to the addition of toxic chemical melamine to feeds in order to artificially inflate protein content. These chemicals can therefore enter the human food chain leading to fatalities and illness

2.4 PATHOGENS THAT HAVE BEEN IMPLICATED IN FISH FEEDS

It has been known that commercial fish feeds are always in contact with the environment, the entire process of processing, handling and storage if not carefully carried out makes it very easy for microorganism and other pathogens to settle on. Some of the pathogens that settle on the feeds release toxins which contaminates the feed and possibly cause spoilage. Contaminated feeds when ingested by fish do not only cause disease to the fish and man, but also increases the levels of histamine, posing a chemical hazard to human health (Bermejo, *et al.,* 2003).

Common pathogens found in fish feeds are highlighted below

1. Bacteria: Bacteria such as the *Escherichia coli, Bacillus species, Staphylococcus species, Streptococcal* species, *Klebsiella* species, *Proteus species and Pseudomonas species* have been reported to contaminate fish feeds (Zmyslowska *et al.,* 1999).

2. Fungi: Fungi are ubiquitous in nature, they have been reported to occur in feed all over the world with some of them capable of producing a wide range of mycotoxins. Fungal contamination of feeds results in

aflatoxicosis. Fungi such as *Aspergillus flavus* and *Aspergillus parasiticus* (molds) are responsible for the production of aflatoxin in feeds. Another mycotoxin that is likely to occur during feed storage and in feed ingredients and in compounded feeds is ochratoxin produced by *Aspergillus* and *Penicillum* species. Furthermore, the *Fusarium* and *Cladosorium* species are also known to occur in feed ingredients, they produce mycotoxins such as deoxynivalenol, zeralenone and fumonisins (Ashley, 1970)

2.5. FEED STORAGE

The following are ways fish feeds can be stored and taken care of to prevent ease of spoilage, infestation by pests and possibly contamination by microorganisms.

i. Bagged feeds should be kept in a cool dry place and kept out of direct sunlight.

ii. Feeds should not be stored for too long i.e not longer than a period of 3 months.

iii. Feed bags should be handled carefully in other to avoid breaking of pellets that will not be consumed by fish.

iv. All moldy feeds should be discarded immediately.

v. Pests such as rats, cockroaches, mice etc. are possible contaminants of feeds hence should be strictly controlled from feed storage room (FAO, 1998).

CHAPTER THREE

3.0 MATERIALS AND METHOD

3.1 STUDY AREA

Fish feed samples were obtained from selected farms in Ikpoba-Okha and Egor local government area in Benin City, Edo state.

3.2 SAMPLE COLLECTION

A total of 12 samples were obtained, six from each aforementioned local government areas. The samples were then transported to microbiology laboratory, University of Benin, in sterile bijou bottles in polythene bags within 1-5 hours of collection of samples.

3.3 BACTERIOLOGICAL ANALYSIS

The bacterial count of the wastewater was determined using pour plate technique. Plate count agar, Bacillus cereus agar, Mannitol salt agar, Salmonella-Shigella agar and Eosin methylene blue agar were used to determine the total heterotrophic bacterial count and coliform count. That is, a ten-fold (10) serial dilution was carried out and appropriate diluents of 0.1 ml sample size were inoculated with pipette into sterile Petri dishes in triplicates. Sterile culture medium was introduced and swirled for mixture. The Petri dishes were allowed to stand for 2-5 mins to solidify and then were inverted to prevent condensation dropping from the lid into the agar and incubated in the incubator at 37°C for 24-48h.

3.4 ENUMERATION

The total heterotrophic bacteria count and coliform count was determined using the formula below;

$$Cfu/ml = \frac{Number\ of\ colonies}{Volume\ of\ inoculum \times Dilution\ factor}$$

3.5 ISOLATION AND IDENTIFICATION OF BACTERIA

Colonies were isolated and purified by repeated sub-culturing for further identification. Distinctive morphological properties of each pure culture such as colony form, elevation of colony and colony margin were observed.

3.6 MORPHOLOGICAL IDENTIFICATION

3.6.1 Gram staining

Smears of the bacterial isolates were prepared and heat fixed on clean grease free-slides. The smears were stained for one minute with crystal violet. This was washed out with distilled water. The slides were flooded with dilute Gram's iodine solution for one minute. This was washed off with distilled water and the smears were decolorized with 95% alcohol for 30 seconds and rinsed off with distilled water. The smears were then counter stained with safranin solution for one

minute. Finally, the slides were washed off with distilled water, air-dried and observed under oil immersion using an objective of ×100 magnification to observe the gram reaction, morphology and arrangement.

3.7 BIOCHEMICAL IDENTIFICATION

3.7.1 Catalase test

Using a sterilized inoculating loop, colonies of the organisms were smeared on the glass slides and a sterile dropping pipette was then used to place a drop of 3% Hydrogen peroxide on the smear. The fluid on the smear was observed for the production of gas bubbles as this is an indication of the presence of catalase. Immediate effervescence of gas indicates the presence of Gram-positive bacteria and this is as a result of hydrogen peroxide being broken down into oxygen and water.

3.7.2 Indole test

Indole test helps to determine the ability of bacterial species to convert tryptophan into Indole. Pure isolated bacterial colony was smeared on a Whatman paper, five drops of Kovac's Indole reagent was added to the Whatman paper. A positive result is indicated by the presence of red or red-violet coloration after 10 sec.

3.7.3 Oxidase test

This test is used to rapidly identify Gram-negative bacteria, in the presence of the enzyme cytochrome oxidase the bacteria is able to convert N, N-dimethyl-p-phenylenediamine oxalate and α-naphthol to indophenol blue. To carry out this test, few drops of 1% aqueous solution of tetramethyl-β-phenylenediamine dihydrochloride (oxidase reagent) were added to a piece of

Whatman filter paper and isolates of pure culture colonies smeared on the paper. A positive result is indicated by the presence of a purple color within 10 sec and a negative result appears yellow.

3.7.4 Citrate utilization test

This test detects the ability of an organism to use citrate as the sole source of carbon and energy. Isolates were picked up using a sterilized wire loop and inoculated onto Simmons citrate agar (SCA) and incubated overnight at 37°C, a citrate positive organism changes color from green to blue.

3.7.5 Urease production test

To determine the ability of a bacterium to produce urease, an enzyme that breaks down urea to release ammonia, the test is done with the bacteria been inoculated onto urea agar base (UAB) supplemented with urea in slants and incubated for 24h at 37^{o}C. A color change of the colorless agar to pink indicates a positive reaction.

3.7.6 Sugar Fermentation Test

Each of the isolates was tested for its ability to ferment a given sugar with the production of acid and gas or acid only. Since most bacteria, especially Gram-negative bacteria utilize different sugars as source of carbon and energy with the production of both acid and gas or acid only, the test is used as an aid in their differentiation. The growth medium used was peptone water and the peptone water was prepared in a conical flask and the indicators; phenol red was added. The mixture was dispensed into test tubes containing Durham tubes. The tubes with their content were sterilized by autoclaving at 121^{0}c for 15 minutes. 1% solution of the sugar was prepared

and sterilized separately at 115^0c for 10 minutes. This was then ascetically dispensed in 5ml volume into the tubes containing the peptone water and indicator. The tubes were inoculated with young culture of the isolates and incubated at 37 ^{0}C. Acid and gas production or acid only were observed after about 24 hours of incubation. Acid production was indicated by the change of the medium from light green to yellow color while gas production was indicated by the presence of gas in the Durham's tubes.

3.8 ANTIBIOTICS SUSCEPTIBILITY TEST

All the bacterial isolates were tested for resistance or sensitivity to different antibiotics using the standard disc diffusion method (Kirby Bauer test). Bacteria were grown between 18-24hr on Mueller-Hinton agar, harvested and then suspended in 0.85% sterile physiological saline solution adjusted to a 0.5 McFarland turbidity standard, corresponding to 108 Cfu/ml. The bacterial isolates were tested against a panel of 8 antibiotics which cut across different classes of antibiotics (1 dose/disc) with varying strengths; Gentamycin (10µg), Ceftriaxone(30µg), Erythromycin (30µg), Cloxacillin (5µg), Ofloxacin (5µg), Augmentin (30µg), Ceftazidine (30µg), Cefuroxime (30µg) . The Antibiotics discs were placed on the surface of each plate by means of antibiotic disc dispenser and incubated at 37°C for 24hours. The results were recorded after 24hr. The diameter of the zone of inhibition around each disc was measured and interpreted as Resistant (R) or Sensitive (S) in accordance with the recommended standard established by the Clinical Laboratory Standards Institute (CLSI, 2011).

CHAPTER FOUR

4.0 RESULT

Socio-cultural perception and understanding of fish farmers towards regular aquaculture practices

Table 4.1 shows the understanding of fish farmers towards regular draining of fishponds. 58.30% of farmers in sampled local government believed that they drain their ponds so the fishes don't die, however, 33.33% regard draining of fishponds as a common practice while 8.33% drains their ponds as a means of monitoring the fish.

Table 4.2 shows the source of water for fish farming in the two sampled local government in Benin metropolis. All (100%) fish farms in both Ikpoba-Okha and Egor local government area make use of borehole as their source of water.

Table 4.3 shows a demographic on the use of antibiotics by fish farmers in Benin metropolis in treatment of fishes and other purposes. 8.33% often use antibiotics, 75% rarely use antibiotics and 16.67% never use antibiotics.

Table 4.4 shows a demographic on the treatment of effluents from fishpond water before discharge into water channels. None (0%) of the fish farmers treat their effluent before discharge.

Table 4.1. Understanding of fish farmer's perspective towards regular draining of fishponds

Location	So they don't die	Common practice	Monitoring the Fish	Others
Egor	**3(50.00)**	**2(33.33)**	**1(16.67)**	**0(0.00)**
Ikpoba-Okha	**4(66.67)**	**2(33.33)**	**0(0.00)**	**0(0.00)**
Total (*n=12*)	**7(58.30)**	**4(33.33)**	**1(8.33)**	**0(0.00)**

Table 4.2: Source of water for fish farming in Benin City

Location	Borehole	Stream/River	Water Tank
Egor	6(100.00)	0(0.00)	0(0.00)
Ikpoba-Okha	6(100.00)	0(0.00)	0(0.00)
Total (*n=12*)	12(100.00)	0(0.00)	0(0.00)

Table 4.3: Use of antibiotics in aquaculture for treatment of fishes/other purposes

Location	Often	Rarely	Never
Egor	1(16.67)	4(66.67)	1(16.67)
Ikpoba-Okha	0(0.00)	5(83.33)	1(16.67)
Total (*n=12*)	1(8.33)	9(75.00)	2(16.67)

Table 4.4: Treatment of effluent from fishponds within Benin Metropolis

Location	Yes	No
Egor	0(0.00)	6(100.00)
Ikpoba-Okha	0(0.00)	6(100.00)
Total (*n=12*)	0(0.00)	12(100.00)

Table 4.5 shows a demographic on the frequency of generation and discharge from fishponds by famers in Egor and Ikpoba-Okha local government areas in Benin metropolis. It was observed that all fish farmers discharge water from fishponds and generate effluents twice weekly, None of the farmers generate effluents weekly and once in two weeks.

Table 4.6 shows a demographic on the final use, discharge and point of disposal of fishpond effluents by famers in Egor and Ikpoba-Okha local government areas in Benin metropolis. It was discovered that 8.33% channel it into irrigation of farmland while 91.67% discharge into surrounding environment. None (0%) of the fish farmers discharge effluents from their fishponds into open water body.

Table 4.7 shows the source of feed for fishes in the ponds. It was observed that all fish farmers used synthetic feed to feed the fishes. None (0%) of the fish farmers make use of poultry droppings.

Table 4.8 The demographic data on type of fish ponds revealed that 66.67% of fish farmers make use of concrete ponds while 33.33% uses earthen pond and none (0%) of them make use of tarpaulin ponds.

Table 4.5: Frequency of effluent generation/discharge from fishponds

Location	Twice weekly	Weekly	Once in two weeks
Egor	**6(100.00)**	**0(0.00)**	**0(0.00)**
Ikpoba-Okha	**6(100.00)**	**0(0.00)**	**0(0.00)**
Total (*n=12*)	**12(100.00)**	**0(0.00)**	**0(0.00)**

Table 4.6: Final use, discharge/point of disposal of fishpond effluent

Location	Farming/Irrigation	Open water body	Surrounding environment
Egor	**1(16.67)**	**0(0.00)**	**5(83.33)**
Ikpoba-Okha	**0(0.00)**	**0(0.00)**	**6(100.00)**
Total (*n=12*)	**1(8.33)**	**0(0.00)**	**11(91.67)**

Table 4.7: Source(s) of feed for fishes in the pond

Location	Poultry Dropping	Synthetic feed	Others
Egor	**0(0.00)**	**6(100.00)**	**0(0.00)**
Ikpoba-Okha	**0(0.00)**	**6(100.00)**	**0(0.00)**
Total (*n=12*)	**0(0.00)**	**12(100.00)**	**0(0.00)**

Table 4.8: Percentages of fishponds sampled during the study in Benin metropolis

Location	Tarpaulin	Concrete	Earthen
Egor	**0(0.00)**	**6(100.00)**	**0(0.00)**
Ikpoba-Okha	**0(0.00)**	**2(33.33)**	**4(66.67)**
Total	**0(0.00)**	**8(66.67)**	**4(33.33)**

Coliform bacteria count as shown in figure 4.1 revealed that fish feeds obtained from farms in Ikpoba-Okha local government area had a higher coliform bacteria count with a logarithmic value of 2.00cfu/ml while feeds from farms in Egor local government area was less with a Logarithmic value of 7.78cfu/ml.

Figure 4.2 shows the bacteria count of fish feeds used by fish farmers in Ikpoba-Okha and Egor local government areas in Benin metropolis. Fish feeds obtained from selected fish farms in Ikpoba-Okha had a lesser bacteria count 17.20cfu/ml in Log_{10} while feeds obtained from farms in Egor local government area had a higher amount of bacteria count in Log_{10} of 52.10cfu/ml.

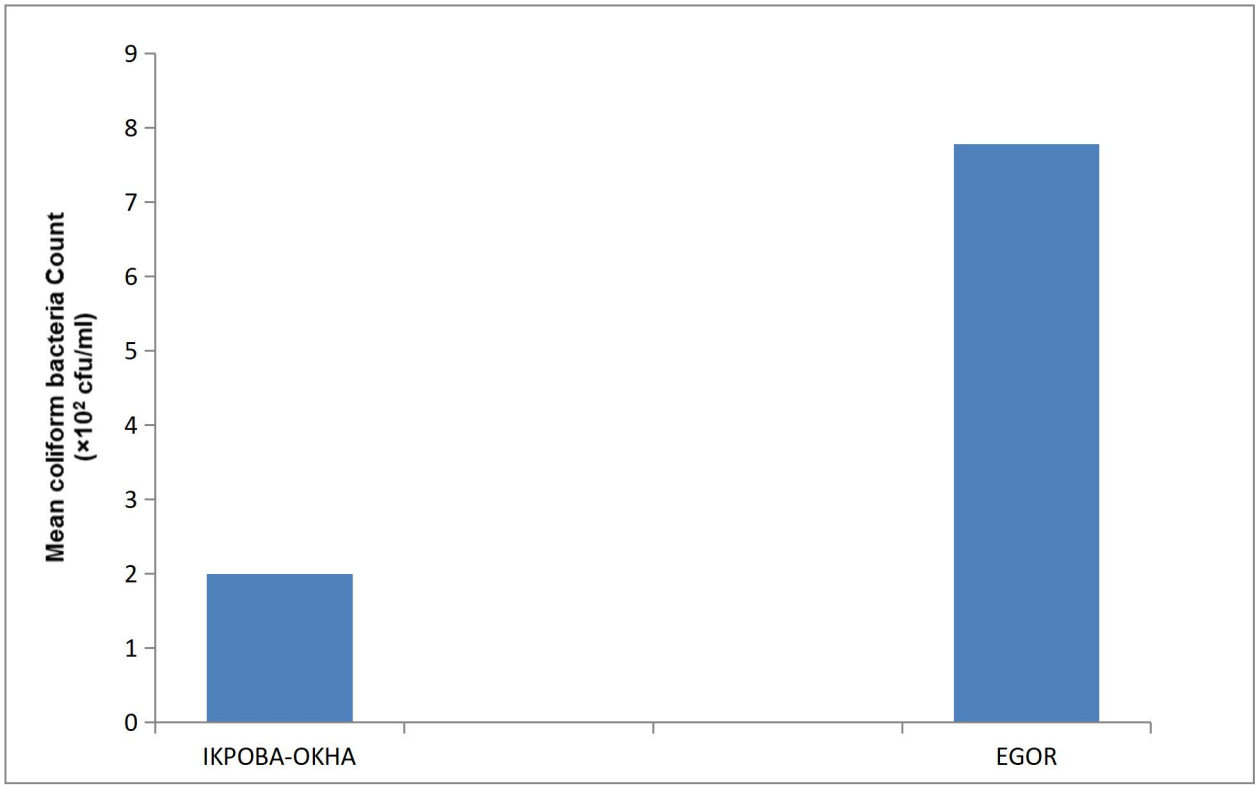

Figure 4.1 shows the mean colifom bacteria count of feed stock samples from Ikpoba-Okha and Egor Local Government Area.

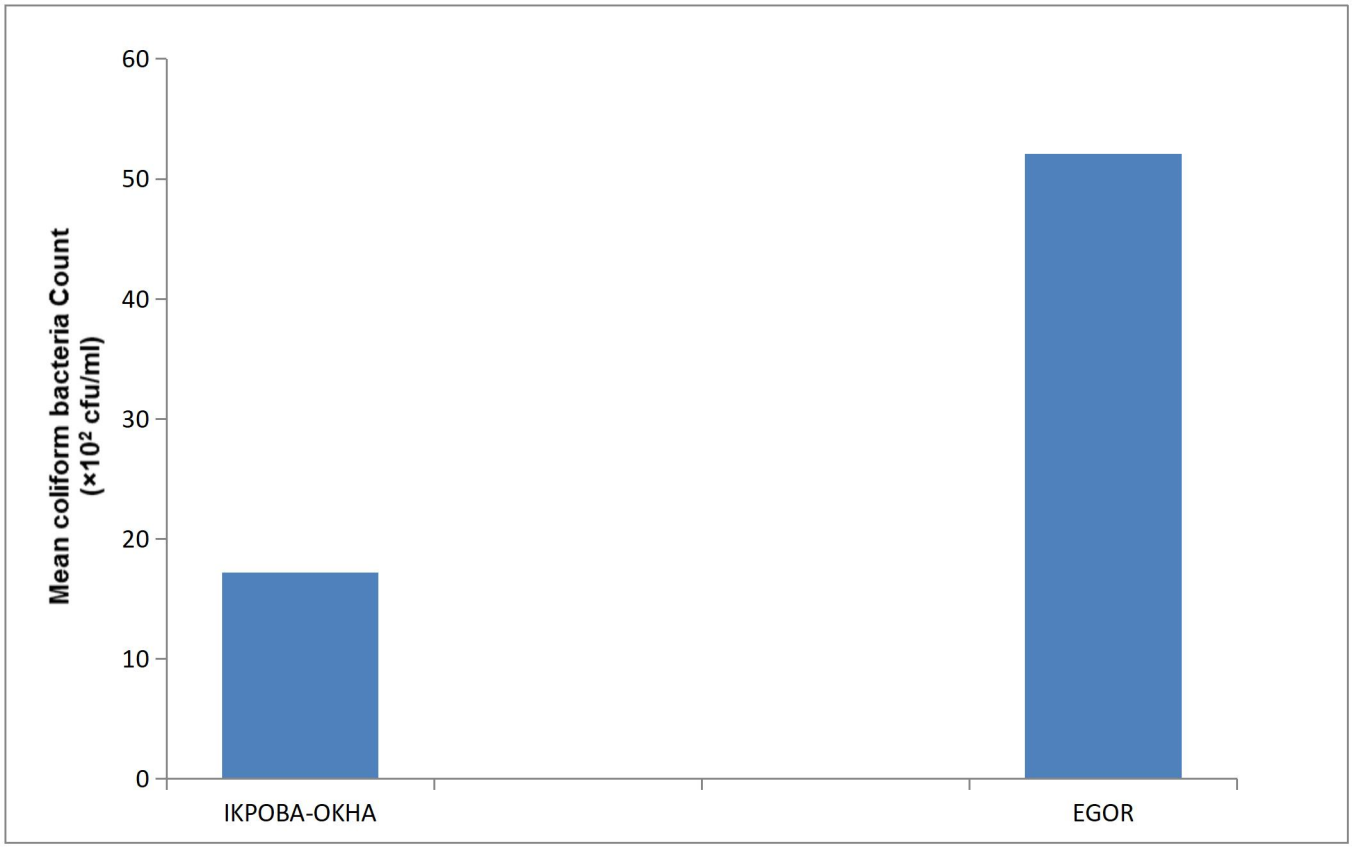

Figure 4.2 shows the mean heterotrophic bacteria count of feed stock samples from Ikpoba-Okha and Egor Local Government Area.

Table 4.9 shows the prevalence of bacterial isolates from feed stock used by fish farmers in Egor and Ikpoba-Okha local government area in Benin metropolis. *Proteus vulgaris* was most prevalent in Egor local government area, while *Escherichia coli* was the most prevalent in Ikpoba-Okha local government area. *Acinetobacter* was least occurrent with a percentage of 2.50% while *Escherichia coli* was the most prevalent with a percentage of 20.00% in both Ikpoba-Okha and Egor LGA combined.

Table 5.0 shows the cultural, morphological and biochemical characteristics of bacteria isolates retrieved from feed stock used by fish farmers in Ikpoba-Okha and Egor local government area. *Citrobacter, Proteus, Enterobacter, Shigella, Salmonella, Klebsiella, Chromobacterium violeceum, Acinetobacter, Escherichia coli, Micrococcus* and *Staphylococcus aereus* were present in the samples

Table 5.1 shows the antibiotic susceptibility pattern of bacteria isolated from the sampled farms in Egor and Ikpoba-Okha local government area in Benin metropolis.

Table 4.9: Prevalence of bacterial isolates in feed stock from Ikpoba-Okha and Egor LGAs in Benin City

Isolate	Ikpoba Okha	Egor	Entire study (%)
Shigella spp	**3**	**-**	**7.50**
Klebsiella spp.	**3**	**2**	**12.50**
Staphylococcus aureus	**-**	**2**	**5.00**
Enterobacter aerogenes	**2**	**1**	**7.50**
Escherichia coli	**5**	**3**	**20.00**
Micrococcus luteus	**2**	**-**	**5.00**
Salmonella	**5**	**2**	**17.50**
Chromobacterium	**2**	**-**	**5.00**
Acinetobacter	**1**	**-**	**2.50**
Proteus vulgaris	**-**	**4**	**10.00**
Citrobacter	**-**	**3**	**7.50**
Total	**23**	**17**	**100.00**

Table 5.0: Cultural, morphological and biochemical characteristics of bacterial isolates from Ikpoba-Okha and Egor LGA

Parameters	**Isolates**										
Cultural characteristics	1	2	3	4	5	6	7	8	9	10	11
Colour	Cream	Cream	Cream	Cream	Grey	Cream	Cream	Grey	Yellow	Cream	White
Shape	Circular	Circular	Circular	Irregular	Circular	Circular	Circular	Circula	Circular	Circular	Circular
Elevation	Convex	Convex	Convex	Effuse	Convex	Convex	Convex	Convex	Convex	Convex	
Margin	Entire	Entire	Entire	Entire	Entire	Entire	Entire	Entire	Entire	Entire	
Size	Small	Large	Small	Small	Small	Small	Small		Small	Small	Small
Morphological characteristics											
Gram stain	-	-	-	-	-	-	-	+	+	-	-
Cell morphology	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Cocci	Cocci	Rod	Rod
Cell arrangement	Single	Single	Single	Single	Single	Single	Single	Clusters	Clusters	Clusters	
Biochemical characteristics											
Catalase	+	+	+	+	+	+	+	+	+	+	+
Indole	+	+	-	-	-	+	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-	-	+	+	-
Citrate	-	-	-	+	+	-	+	+	-	+	+
H_2S production	-	-	+	-	+	-	+	-	-	-	-
Glucose	+	+	+	+	+	+		+	+	+	+
Lactose	+	+	-	+	-	-	+	-	-	-	-
Sucrose											
Mannitol	+	+	+	+	-	+	+	+	+	-	-
Gr. Diff.Agar	Green metallic Sheen (EMB)	Black spot (SSA)		Black spot (SSA)	Straw (SSA)			Yellow (MSA)			
Probable Identity	Escherichia *coli*	*Salmonella*	*Klebsiella*	*Proteus*	*Shigella*	*Citrobacter*	*Enterobacter*	*S.aureus*	*M. luteus*	*Chromobacterium*	*Acinetobacter*

Key: + = Positive, - = Negative, Gr.Diff.Agar = Growth on differential agar, MSA= Mannitol salt agar, *S.aureus=Staphylococcus aureus, M.luteus= Micrococcus luteus*

Table 5.1: Antibiotics susceptibility pattern of bacteria isolates

		GEN	CTR	ERY	CXC	OFL	AUG	CAZ	CAX
Escherichia coli	4	S	R	R	R	S	R	R	R
Salmonella	6	S	R	R	R	R	R	R	R
Klebsiella	9	R	R	R	R	S	R	R	R
Proteus	19	S	R	R	R	S	R	R	R
Shigella	2	S	R	R	R	S	R	R	R
Citrobacter	5	R	R	R	R	S	R	R	R
Enterobacter		S	R	R	R	S	R	R	S
Staphylococcus aureus		S	R	R	R	S	R	R	R

Key: R=Resistant, S=Susceptible, GEN=Gentamycin, CTR=Ceftriaxone, ERY=Erythromycin, CXC=Cloxacillin, OFL=Ofloxacin, AUG=Augmetin, CAZ=Ceftazidine, CAX=Cefruroxime

CHAPTER FIVE

5.0 DISCUSSION

The analysis carried out on commercial, otherwise called synthetic fish feeds as obtained from various fish farms in Ikpoba-Okha and Egor local government area of Benin metropolis showed contamination of the feeds by some pathogenic bacteria, as this could be as a result of environmental condition during processing, handling, transport or/and storage (FAO, 1998).

Socio-cultural demographics on perception and understanding of fish farmers towards regular aquaculture practices was obtained during sample collection. From the response of the farmers, it was documented that most of them in percentage value(%) of 58.30 drains their ponds so as to reduce mortality rate of fish, 33.33 only carry out draining of fishponds as they believe it to be a common practice, meanwhile 8.30 perform the task as a way of monitoring the fish. The greater percentage of the findings is similar to the one carried out by Adeogun *et al.*(2007) where it was observed that 48.2% of fish farmers in Lagos State drained their ponds via the flow through system technique. In contrast to this study, Adeogun *et al.*(2007) documented that 35.7% of farmers do not drain their ponds at all, however water recirculatory was prominent with 16.1% of the farmers.

It was recorded that all fish farmers in both sampled local government area in Benin metropolis make use of synthetic feeds only, which is in close similarity with field survey carried out by

Ifejika *et al.* (2013). It was deduced that 97.3% of farmers operating in Niger State, Nigeria make use of synthetic feeds as nine different feed brands were sampled.

In a research done by Adeogun *et al.* (2007), It was documented that 62.5% of farmers used concrete ponds to rear fish, while 21.4% made use of earthen ponds. This is very similar to the field survey carried out during the course of this study, where it was noted that 66.67% of farmers made use of concrete ponds, while 33.33% made use of earthen ponds, meanwhile there was no record of farmers using tarpaulin pond. This shows that a lower percentage of farmers in the sampled locations used earthen ponds rather than concrete ponds.

According to the survey carried out, all (100%) fish farmers depend on borehole as water source for their fish ponds. This may be due to fact that borehole system is one of the commonest and easiest water source in Benin City. This result is however different from the one obtained by Olaoye *et al.*(2013) on the assessment of socio-economic analysis of fish farming in Oyo metropolis, where most (61.7%) of farmers depend directly on stream or river as a major source of water, 25.2% rely on deep well while only 13.1% depend on borehole.

Bacteriological analysis of the study revealed that *Escherichia coli* was most prevalent in both sampled local government area, with percentage(%) of 20.00. This may be due to storage and temperature condition surrounding the feeds. The result is in accordance with the analysis carried out by A.A Nwabueze and E.O Nwabueze (2011) in their study on microbial flora of fish feeds sold in Asaba, Southern Nigeria, observed that *Escherichia coli* is the most dominant microorganism found in synthetic fish feeds sold in Asaba.

Cultural, morphological and biochemical characteristics of bacterial isolates showed a total of eleven isolates identified as *Shigella* sp*, Staphylococcus aureus, Enterobacter aerogenes, Escherichia coli, Micrococcus luteus, Salmonella, Chromobacterium, Acinetobacter, Proteus*

vulgaris, Citrobacter, and *Klebsiella* sp.The frequency of occurrence of *Escherichia coli* was seen to be more than the other bacterial isolates in the feed samples which is not in accordance with the work done by (Olayiwola *et al.,* 2015) that reported a higher occurrence of *Bacillus* sp in fish feed. The presence of *Escherichia coli* suggest contamination most probably from feed retailers while the presence of *Staphylococcus aureus* in the feeds suggest contamination from market sellers (Ubeiebi, 2017). The presence of these organisms reveals a poor state of hygiene employed in production, packaging, transportation, handling and storage of fish feeds.

The Gram positive bacteria identified in this study was susceptible to gentamycin and ofloxacin, meanwhile all bacteria identified in this study were resistant to ceftriaxone/ceftrazone which is contrary to the study done by (Olayiwola *et al.,* 2015) where all Gram negative bacteria were susceptible to ceftriaxone/ceftrazone.

5.2 CONCLUSION

From the results and findings in this study, it can be concluded that fish feeds are being constantly exposed to the environment, from the point of manufacturing to when it reaches the farmers. This revealed that contamination is certain to occur. Contaminated feeds are not only unsafe for consumption by fish but can also be detrimental to the health of human who consumed such fish. Therefore, factories that manufacture fish feeds should follow standard process, farmers and sellers should follow proper hygiene when handling and storing feeds so as to ensure pathogen-free fish feed.

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APPENDIX I

EQUIPMENTS AND MATERIALS USED

Bunsen burner - for sterilization

Syringe - for collection of water samples

Petri dishes – for culturing of microorganisms

Pipettes – used for transferring water samples

Conical flasks – used for storing the media

Colton wool – for mopping up liquid, cleaning slides, etc

Autoclave – for sterilizing materials like petri dishes

Hot air oven – used for incubation

Refrigerator – for storing specimen and media

Microscope – used for viewing/identification of microorganisms

Incubator – used for incubating at different temperatures.

APPENDIX II

MEDIA COMPOSITION

Nutrient agar

Meat peptone	5g
Beef extract	3g
Sodium chloride	5g
Agar	15g
Water	1L

Maconkey agar

Peptone (pancreatic digest of gelatin)	17g
Proteose peptone (meat and casein)	3g
Lactose monohydrate	10g
Bile salts	1.5g
Sodium chloride	5g
Neutral red	0.03g
Crystal violet	0.001g
Agar	13.5g
Distilled water	1L

Mueller-hinton agar

Beef	300g
Casein acid hydrolysate	17.5g
Agar	17g

Starch	1.5g

Simmons citrate agar

Sodium chloride	5g
Sodium citrate (dehydrate)	2g
Ammonium dihydrogen phosphate	1g
Magnesium sulphate (heptahydrate)	0.2g
Bromothymol blue	0.08g
Agar	15g
Distilled water	1L

Indole test (Kovac's reagent)

P-dimethylaminocinnamaldehyde	10g
Hydrochloric acid 37%	100ml
Distilled water	900ml

Oxidase test

1% tetra-methyl-p-phenylenediaminedihydrochloride mixed in distilled water.

Catalase test

Drops of 3% hydrogen peroxide on a smear of a single colony on a glass slide

GRAM STAINING

Crystal violet

Crystal violet	2g
95% ethyl alcohol	20ml
Ammonium oxalate monohydrate	0.8g
Distilled water	

Iodine

Iodine	1g
Potassium iodine	2g
Sodium bicarbonate	3g
Water	300ml

Alcohol

95% ethanol

95% acetone

Safranin

Safranin	2.5g
Distilled water	1L

APPENDIX III

Table for figure 4.1: shows the mean coliform bacteria count of feed stock samples from Ikpoba-Okha and Egor Local Government Area.

PARAMETER	IKPOBA OKHA($\times 10^3$ cfu/ml)	EGOR($\times 10^3$ cfu/ml)
F1	0 ± 0.00^a	0.8 ± 3.27^c
F2	0.15 ± 1.22^a	0.5 ± 2.04^b
F3	0 ± 0.00^a	0 ± 0.00^a
F4	0.2 ± 1.63^a	1.35 ± 5.51^d
F5	0.5 ± 2.04^a	1.3 ± 5.31^d
F6	0.35 ± 1.42^a	0.7 ± 1.83^a

Table for figure 4.2: shows the mean heterotrophic bacteria count of feed stock samples from Ikpoba-Okha and Egor Local Government Area.

PARAMETER	IKPOBA-OKHA ($\times 10^3$ cfu/ml)	EGOR($\times 10^3$ cfu/ml)
F1	1.35 ± 5.51^a	4.85 ± 19.67^d
F2	0.85 ± 3.47^a	6.1 ± 24.90^d
F3	1.05 ± 4.28^a	5.2 ± 21.22^d
F4	1.35 ± 5.50^b	7 ± 28.57^d
F5	3.85 ± 15.71^a	4.2 ± 17.15^c
F6	1.9 ± 7.75^a	3.9 ± 15.92^d