Assessment of Antibiotic Residues in Commercial Aquaculture Feeds in Selected Farms in Awka, Anambra State, Nigeria

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Abstract— *Aquaculture feeds are often compounded with antibiotics to promote growth and prevent diseases in fish. However, the use of antibiotics in aquaculture feeds has raised concerns about the presence of antibiotic residues in fish which subsequently leads to antimicrobial resistance. This study therefore investigated antibiotic residues in Nine (9) different fish feeds used in three selected fish farms located in Awka, Anambra state, Nigeria. One gram (1g) of the feed samples were used for antibiotics residues analysis. The fish feed samples were collected in three replicates each, making it twenty-seven treatment samples. The targeted antibiotics were the commonly used antibiotics which include Tetracycline, Ciprofloxacin, Amoxicillin and Azithromicin. The feed samples were analyzed for antibiotics residues concentration using Uv-Visible Spectrophotometry. The results showed that the mean antibiotic residue concentration from the three farms were higher in the following order; Tetracycline > Ciprofloxacin > Amoxicillin > Azithromicin, with mean concentrations of 85.40±1.78 ug/g, 0.031±0.002 ug/g, 0.019±0.002 ug/g and 0.010±0.001 ug/g respectively. The mean concentration of Tetracycline was higher than the European Union (EU) maximum residue limit of 0.1 ug/g but Ciprofloxacin, Amoxicillin and Azithromicin were all below the EU maximum residue limits of 0.05 ug/g in feed. This study concluded that the analyzed feed samples contained low dose of antibiotics which may contribute to the development of antibiotic-resistant bacteria. It is therefore recommended that antibiotics should not be used in fish feed formulation, so as to help the farmer to make responsible use of antibiotics when there is a genuine need.*

Keywords*— *Antibiotics resistance, Drug residues, Aquaculture, Fish feeds, Awka-Nigeria.

I. INTRODUCTION

Nutrition is a major factor determining the potential of cultured fish to exhibit their genetic capability for growth and reproduction. The increasing costs and limited availability of fish feed have prompted the need for research into alternative feed sources (Ogunkalu, 2019). It is essential to provide cultured fish with sufficient nutritional feed to enhance growth and immunity, ultimately leading to a successful harvest. In order to meet the nutritional requirements of cultured fish, fish feeds are often enriched with additives. Feed additives are dietary ingredients that are incorporated into feed formulations not only for the usual provision of basic nutritional requirements as offered by traditional feed, but also to improve growth, immune responses, and disease resistance in aquatic animals (Onomu and Okuthe, 2024). These additives include probiotics, prebiotics, enzymes, antibiotics, medicinal plants and other feed additives. Antibiotics can be used as feed additives in animal production in order to maintain animal health (Ghimpețeanu *et al.*, 2022). In the aquaculture industry, the health of aquatic animals is of utmost importance in order to ensure optimal production and profitability. Therefore antibiotics have been commonly used to combat diseases and parasites in aquatic animals (Lulijwa *et al*., 2020). Antibiotics have been included in aquatic feed to prevent or treat bacterial diseases and promote growth (Onomu and Okuthe, 2024). The most often used antibiotics in aquaculture are oxytetracycline, florfenicol, macrolides, fluoroquinolones, and sulfadiazine (Lulijwa *et al*., 2020). The most common route for the administration of antibiotics in aquaculture is through aquaculture feed (Liu *et al*., 2017). Pathmalal (2018) reported that antimicrobials fed to fish are not efficiently metabolized by fish and are eliminated through urine and feces into the pond water. While antibiotics have been effective in controlling diseases, their use has raised significant concerns regarding fish health, environmental impact, and human health (Chowdhury *et al.*, 2022). A consequence of the use of the antibiotics in food-producing animals is the presence of drug residues, even in a very low concentrations, in the edible tissues of the treated animal (Monteiro *et al*., 2018). The presence of antibiotic residues in aquaculture products could result in the development of bacterial resistance and toxicity to consumers that can lead to morbidity and/or death (Okocha *et al.*, 2018). Consumption of such products may result in many health problems in humans. Chiefly among the health concerns is the development and propagation of antimicrobial resistance along the food chain (Tang *et al*., 2020). Several studies have repprted the presence of antibiotic residues in various types of animal feed (Manyi-Loh *et al.,* 2023). Currently, more antibiotics are used in food animal production to promote growth and prevent disease than in the entire human population. Although millions of human lives have benefited from antimicrobials, the majority (73%) of such antimicrobials are those utilised for animal production (Van Boeckel *et al.*, 2017). Among intensive types of livestock production that use antimicrobial agents, particular attention should be paid to fish farms because of their direct impact on the aquatic environment (Shao *et al*., 2021). Antibiotics in fish culture are usually administered through feed and the intestinal environment of fish is an optimal site for the selection of antibiotic resistant bacteria and through fish feces, antibiotic resistant bacteria are dispersed to the water column or sediments (Bajorski *et al.*, 2020). Thus, utilising a diverse range of these antimicrobial agents in fish feed, including those used in human medicine, guarantees their persistence in the aquatic environment for an extended period (Okon *et al*., 2022) which could lead to the development of antimicrobial resistance. Large quantities of antimicrobials are used in aquaculture feed in low- and middle-income countries, often without professional supervision with consequences for development and spread of resistance and global public health.

Okeke *et al*. (2022) reported that antibiotic usage increased from 21.1 to 34.8 billion defined daily doses in 76 countries between 2000 and 2015, and it is anticipated to reach 126 billion defined daily doses in 2030 if no policy changes are made. There are strict laws on the usage of antibiotics and chemotherapeutics in the aquaculture feed industry (Bojarski *et al.,* 2020). In the absence of effective regulations, there is a risk for large number of antibiotics including medically important antibiotics (Chowdhury *et al.*, 2022) becoming available for use in fish feed formulation. The United States Food and Drug Administration (FDA) enforces rigorous monitoring practices for antibiotics, such as oxytetracycline and florfenicol, with a zero-tolerance policy for certain high-risk residues (Miranda *et al*., 2018). Meanwhile, antibiotics used in aquaculture feed help to improve fish health by killing or inhibiting the growth of pathogenic bacteria. On the other hand, it causes more side effects. Antibiotics have helped to reduce mortality rates in developing nations by reducing deaths from infections, but the costs of antibiotic contamination continue to be a major issue (Okeke *et al*., 2022). In order to ensure food safety for consumers, more and more studies have attempted to find effective and rapid methods for the detection of antibiotic residues in feed and food products (Long *et al*., 2018). Consumers are also increasingly interested in consuming quality food, and are turning to organic products, which provide them with more safety relative to conventional products (Ghimpețeanu *et al.,* 2022). Therefore, this study was aimed to assess the levels of antibiotics in commercial aquaculture feeds used in selected fish farms in Awka to ensure the safety of aquaculture products and protect human health.

II. MATERIAL AND METHODS

2.1 Sample Collection and Preparation:

A total number of nine (9) different fish feeds samples were collected from three different fish farms in Awka, Awka South local government Area, Anambra state. Awka south is located within Latitude 6°21'N to 6°08'N, and between Longitude 7°07'N to 7°44'E. The study sites where the fish feed samples were collected are Fish Farm 1 (latitude 7°48'33''N, Longitude 6°91'75''E), with fish feed samples (A,B,C), Fish Farm 2 (Latitude 6°21'06''N, Longitude 7°07'60''E) with fish feed samples (D,E,F) and Fish Farm 3 (Latitude 6°21'06''N, Longitude 7°07'09''E) (Source Google), with fish feed samples (G,H,I). The fish feed samples were collected in three replicates each, making it twenty-seven treatment samples. The fish feed samples collected depends on the type of fish feed used by those fish farmers. 9mm feed samples was used for this analysis. The feed samples were collected in clean plastic bags and taken to Springboard laboratory for analysis. Springboard Laboratory is located at Udoka Estate in Awka, Anambra state.

2.2 Preparation and Methods:

2.2.1 Antibiotics Assay:

One gram of the feed samples was weighed with digital weighing balance (Model OHAUS M44Y) and then grounded using a mortar and pestle. Tetracycline was extracted with Mcllvaine buffer, Quinolones was extracted with 0.1% formic acid in acetonitrile, Beta-Lactams were extracted with a solution consisting of 0.1 M Phosphate buffer (pH 7) and methanol (1:1) while Macrolides were extracted using 0.1 M Phosphate buffer (pH 6.9), to enhance their solubility. After extraction, Tetracycline was vortexed and then centrifuged before determining the absorbance at 355 nm, Quinolones were vortexed and then centrifuged before determining the absorbance at 278 nm, Beta-Lactams were vortexed and then centrifuged before determining the absorbance at 230 nm, and Macrolides were vortexed and then centrifuged before determining the absorbance at 210 nm using a search tech UV-Visible spectrophotometer as described by Granados-Chinchilla and Rodríguez (2017). Tetracycline standards were prepared by using a gradient of known concentrations of tetracycline. The results were represented in micrograms of Tetracycline per gram of feed (ug TET/g). Similarly, Ciprofloxacin standards were prepared using a gradient of known concentrations of Ciprofloxacin, with results represented in micrograms of Ciprofloxacin equivalent per gram of feed (ug CIPRO Eq/g). Amoxicillin standards were also prepared using a gradient of known concentrations of Amoxicillin, with results represented in micrograms of Amoxicillin equivalent per gram of feed (ug AMOX Eq/g). Lastly, Azithromycin standards were prepared using a gradient of known concentrations of Azithromycin, with results represented in micrograms of Azithromycin equivalent per gram of feed (ug AZITHRO Eq/g).

2.2.2 Farm Record Data Collection

In this study, farm records from the three different fish farms were collected to assess the level of antibiotics residues in the feeds consumed by the fish over a production cycle of 6 months. Farm 1 stocked 2000 catfish and fed them with 2400kg of feed, which is equivalent to 160 bags. Farm 2, on the other hand, stocked 3000 catfish and fed them with 3600kg of feed, equivalent to 240 bags. Lastly, Farm 3 stocked 2500 catfish and fed them with 3000kg of feed, equivalent to 200 bags.

2.3 Data Analysis:

Data obtained from this study were expressed as mean ± SEM and subjected to One-way Analysis of Variance (ANOVA) using the IBM Statistical package for Social Science (SPSS) version 29.0.2, Differences were considered significant at $p<0.05$.

III. RESULTS AND DISCUSSION

3.1 Antibiotic Residues in the nine (9) Fish Feed Samples from the Three Fish Farms:

The results of the antibiotics residues in the sampled fish feeds are shown in Table 1. The results revealed that all the nine fish feed samples from the three different fish farms contained varying concentrations of commonly used antibiotics. Antibiotics detected in the feed samples were Tetracycline, Ciprofloxacin, Amoxicillin and Azithromycin which are widely used in aquaculture. The results showed that the mean antibiotic residue concentrations were higher in the order; tetracycline > ciprofloxacin > amoxicillin > azithromycin with mean concentrations of 85.40±1.78 ug/g, 0.031±0.002 ug/g, 0.019±0.002 ug/g and 0.010±0.001 ug/g respectively. In this study, Tetracycline residue were the highest among the antibiotics tested. Samples G and H contained the highest levels of Tetracycline residues and sample I contained the lowest level of Tetracycline residue level. However, sample D contained the highest level of Quinolone residue level while sample I contained the lowest level of Quinolone residue level. However, sample F contained the highest level of Beta-lactam residue level while sample C contained the lowest level of Beta-lactam residues. Sample E contained the highest level of Macrolide residue level while sample I contained the lowest level of Macrolide residue level.

TABLE 1
THE LEVELS OF ANTIBIOTICS DETECTED IN THE NINE (9) FISH FEED SAMPLE

Treatment	Tetracycline residue (ug TeT Eq/g)	Quinolone residues (ug CIPRO Eq/g)	Beta-lactams residues (ug AMOX Eq/g)	Macrolides residues (ug AZITHRO Eq/g)	Total antibiotics residue
Sample A	87.46± 0.08	0.030± 0.05	0.013±0.01	0.007±0.03	87.51±0.14
Sample B	87.79± 0.01	0.032± 0.02	0.026±0.01	0.013±0.06	87.86±0.01
Sample C	84.85± 0.08	0.027±0.08	0.010±0.03	0.007±0.09	84.89±0.17
Sample D	82.60± 1.91	0.042± 0.01	0.013±0.05	0.006±0.04	82.66±1.93
Sample E	78.63 ±1.91	0.029± 0.03	0.016±0.04	0.019±0.02	78.69±1.93
Sample F	87.37 ±1.38	0.036±0.02	0.032 ±0.02	0.009±0.05	87.44±1.41
Sample G	92.45 ±0.48	0.033±0.05	0.022±0.02	0.016±0.06	92.52±0.50
Sample H	90.92 ±1.03	0.029±0.01	0.019±0.01	0.012±0.05	90.98±1.07
Sample I	76.51 ±1.52	0.022±0.02	0.018±0.01	0.002±0.01	76.55±1.54
Mean	85.40±1.78	0.031±0.002	0.019±0.002	0.010±0.001	
MRL Values by (EU)	0.1ug/g	0.05ug/g	0.05ug/g	0.05ug/g	
MRL = Maximum Residues Limit; EU = European Union (2010).					

MRL = Maximum Residues Limit; EU = European Union (2010)

It was also observed in Table 1 that tetracycline had the highest residue concentrations in all the analyzed fish feed samples and this may be as a result of its frequent use in aquacuture feed while macrolide (Azithromycin) had the lowest residue concentrations. Tetracyclines are widely used for both the treatment and prevention of diseases in animals as well as for the promotion of rapid animal growth and weight gain (Pérez-Rodríguez *et al*., 2018).The antibiotic type detected in the fish feeds were very similar to those reported by Tang *et al*., (2020) for cultured fish in China indicating that antibiotics were used in aquaculture production. Antibiotics are commonly used in animal husbandry as growth promoters, with Tetracyclines being one of the most widely utilized antibiotic. Tetracyclines are of particular importance due to their extensive use in the industry, surpassing the quantities applied of almost every other antibiotic group (Granados-Chinchilla and Rodríguez, 2017). Tetracyclines are a family of compounds frequently employed due to their broad spectrum of activity as well as their low cost, compared with other antibiotics. However, Lui *et al*. (2017) reported that macrolides are also commonly used as feed additives in fish cultivation for growth promotion and disease prevention and treatment. The mean concentrations of Tetracycline were higher than the maximum residue limit (MRL) while Ciprofloxacin, Amoxicillin and Azithromycin observed in the fish feed samples were all below the MRL as shown in Table 1. Antibiotics can cause serious effects on human health which have led to the introduction of maximum residue limits (MRL) in food safety legislation (EU, 2019). The wide and sometimes inappropriate uses of antibiotics in aquaculture feed have resulted in the presence of the drug residue above the maximum residue limits (MRLs) in fish produced with potential health implications such as nausea, sore throat, diarrhoea and serious illnesses (Okocha *et al*., 2018). Furthermore, the lack of Maximum Residue Limits (MRLs) for some antibiotics complicates the assessment of antibiotic residues in aquaculture products. The results of this study can serve as a reference for the development of quality standards for aquatic products, particularly in the absence of established MRLs for certain antibiotics. It is important for fish farmers and feed manufacturers to adhere to MRLs to ensure the safety of consumers and to comply with regulatory requirements. Monitoring and testing of fish feeds for antibiotic residues should be conducted regularly to ensure compliance with MRLs and to prevent the presence of harmful residues in fish products.

TABLE 2
MEAN ANTIBIOTICS RESIDUES IN FARM 1, FARM 2, AND FARM 3, IN DIFFERENT FEED SAMPLES FOR ONE PRODUCTION CYCLE OF 6 MONTHS

Sample Sites	Samples	Tetracycline	Ciprofloxacin	Amoxicillin	Azithromicin
	(Treatment)	(ug/g)	(ug/g)	(ug/g)	(ug/g)
Farm 1	A	209.09±1.01	0.72±0.31	0.0312±0.03	0.0168±0.01
	B	198.24±0.23	0.1008±0.12	0.0312±0.03	0.0144±0.07
	C	188.712±0.32	0.0696±0.01	0.0384±0.05	0.0456±0.02
Farm 2	D	254.55+1.32	0.081±0.03	0.03±0.03	0.021±0.05
	E	262.11±1.44	0.108±0.05	0.096±0.04	0.027±0.03
	F	277.35±0.45	0.099±0.07	0.066±0.03	0.048±0.04
Farm 3	G	316.008±1.32	0.115±0.16	0.094±0.04	0.047±0.03
	H	327.312±1.97	0.1044±0.12	0.0684±0.07	0.0432±0.02
	I	275.436±0.54	0.0792±0.04	0.0648±0.01	0.0072±0.03

Tables 2 shows the concentration of antibiotics that cultured fish consumed in six months of production cycle. The presence of antibiotics residues in feed samples observed in this study posed a significant risk to the quality and safety of fish. The finding of this study suggested that these feed samples should not be used for a long period in fish production, as they may lead to the accumulation of antibiotics in cultured fish. The continuous ingestion of antibiotic-laden feed enhances build-up of drugs residues in edible tissues of fish. Long-term exposure of fish to feed containing antibiotics could be systemically toxic to consumers and has a direct negative impact on the complex microflora that inhabits the human gastrointestinal system with potentially adverse implications (Monteiro and Andrade, 2018). The consumption of such fish may result in human health risks and conditions such as drug hypersensitivity reactions, the disruption of normal intestinal flora and carcinogenic, mutagenic and teratogenic effects (Okocha *et al*., 2018). The daily consumption of aquatic products may lead to exposure to various levels of low-dose antibiotics which can lead to antimicrobial resistance in fish. The presence of antibiotics residues in fish feeds is a major concern as it can contribute to the development and transmission of antibiotic-resistant bacteria through the food chain (Chowdhury *et al.,* 2022). Antibiotic resistance is of great public health concern because the antibiotic-resistant bacteria associated with fish may be pathogenic to humans, easily transmitted to humans via food chains, and widely disseminated in the environment via their wastes (Manyi-Loh *et al.*, 2023). These may cause complicated, untreatable, and prolonged infections in humans, leading to higher healthcare cost and sometimes death. Residues of antimicrobials in feed have received much attention in recent years because of growing food safety and public health concerns. Their presence in food of animal origin constitutes socioeconomic challenges in international trade in animal and animal products (Lee *et al*., 2023). The major public health significance of antimicrobial residues includes the development of antimicrobial drug resistance, hypersensitivity reaction, carcinogenicity, mutagenicity, teratogenicity, bone marrow depression, and disruption of normal intestinal flora. In order to ensure food safety and avoid exposure to these substances, national and international regulatory agencies have established tolerance levels for authorized veterinary drugs (Pérez-Rodríguez *et al*., 2018). In order to ensure the correct usage and dosage of antibiotics in food and feedstuffs (Granados-Chinchilla and Rodríguez, 2017), the accurate assessment of antibiotic residues is essential for determining the levels of these substances in feed and food products, as well as for evaluating potential risks to human health.

TABLE 3
MEAN ANTIBIOTICS RESIDUES IN FARM 1, FARM 2 AND FARM 3 IN DIFFERENT SAMPLES OF FEED THAT A FISH WILL CONSUME FOR A PRODUCTION CYCLE OF 6 MONTHS

Sample sites	Samples (Treatment)	Tetracycline (ug/g)	Ciprofloxacin (ug/g)	Amoxicillin (ug/g)	Azithromicin (ug/g)
Farm 1	A	0.105±0.03	0.000036±0.02	0.000016±0.08	0.000084±0.02
	B	0.099±0.05	0.000052±0.05	0.000016±0.08	0.000072±0.04
	C	0.094±0.07	0.000034±0.01	0.000019±0.05	0.000022±0.05
Farm 2	D	0.105±0.14	0.000052±0.09	0.000031±0.03	0.000016±0.01
	E	0.109±0.16	0.000034±0.01	0.000023±0.09	0.000014±0.02
	F	0.092±0.05	0.000024±0.02	0.000021±0.03	0.000024±0.06
Farm 3	G	0.102±0.15	0.000032±0.03	0.000012±0.04	0.000084±0.02
	H	0.105±0.17	0.000043±0.06	0.000038±0.07	0.000018±0.05
	I	0.111±0.13	0.000039±0.05	0.000026±0.01	0.000019±0.07

The levels of antibiotics detected in the feeds were relatively low, however, the daily consumption of these feeds by the fish as shown in Table 3 and can lead to the accumulation of antibiotics in their tissues. This continuous exposure to antibiotics can potentially result in the development of antibiotic resistance in the fish and in the environment. In many countries, aquaculture production systems are not separated from the environment leading to the accumulation of antimicrobial residues in the waters used for fish farming and adjacent waters affecting wild fish, plankton and sediments (Lulijwa *et al.*, 2020). Yang *et al*. (2021) reported that antibiotics have become widespread in the environment due to their extensive and long-term use, influencing both human health and environment, due to the emergence of antibiotic resistance. Studies by Erofeeva *et al* (2021) have shown that a significant percentage of all manufacturers add antibiotics to healthy animals' feed to prevent, rather than to cure diseases. The presence of antibiotics residues in fish feeds is a major concern as it can contribute to the development of antibiotic-resistant bacteria (Ghimpețeanu *et al*., 2022). A number of factors can determine the best alternatives to antibiotics to be used within an aquaculture system especially when compared to FAO/WHO and European Union maximum acceptable limit. Good animal husbandry practices as well as the use of alternatives to antibiotics such as vaccination, probiotics, phage therapy, and essential oils are recommended panaceas to reducing the use of antimicrobial residues in aquaculture feed (Okocha *et al.*, 2018). Strategies to reduce or limit the therapeutic use of antibiotics in animals through improved animal nutrition, improved living conditions and waste management, biosecurity measures, and improvement in animals' natural immunity can result in infection prevention and control (Manyi-Loh *et al.,* 2023). Ultimately, the use of antibiotics in food-producing animals should be limited by incorporating possible alternative substances, including probiotics, prebiotics, and plant-derived or crude plant extracts for the treatment and prevention of diseases (Guetiya-Wadoum *et al.,* 2016). To assure food safety, continuous monitoring and identification of risk factors in aquatic feeds and products are crucial, particularly in underdeveloping and developing nations. This therefore calls for strengthening of regulations that direct antibiotic manufacture, distribution, dispensing, and prescription, hence fostering antibiotic stewardship (Manyi-Loh *et al.,* 2023). Feed manufacturers and authorities should establish procedures and instructions for the effective and safe use of authorized and prescribed veterinary medicinal products. EU (2019) established rules for the authorization of use of veterinary medicinal products such as antibiotics in feeding stuffs, including the manufacture, distribution, advertising, and surveillance of such products. Feed business operators, which handle manufacturing, storing, transporting, or placing medicated feed and intermediate products on the market, must be authorized by the competent authority, in accordance with the authorization system, to ensure both the safety of the feed and the traceability of the products (Ghimpețeanu *et al*., 2022).

IV. CONCLUSION AND RECOMMENDATION

Antibiotics residues in fish feeds used for aquaculture have become a growing concern due to their potential impact on human health and the environment. This study evaluated the levels of antibiotics residues in nine fish feeds used in three aquaculture farms in Awka, Anambra State. The results revealed that all the nine fish feed samples from the three different fish farms contained varying concentrations of commonly used antibiotics. The result showed that the mean antibiotic residue concentrations were higher in the order; Tetracycline >Ciprofloxacin > Amoxicillin > Azithromycin. It was also observed that

Tetracycline had the highest residue concentrations in all the analyzed fish feed samples and this may be as a result of its frequent use in aquacuture feed while Azithromycin had the lowest residue concentrations. The mean concentrations of Tetracycline were higher than the maximum residue limit (MRL) while Ciprofloxacin, Amoxicillin and Azithromycin observed in the fish feed samples were below the MRL. To address this issue, it is recommended that fish farmers should carefully monitor the amount of antibiotics in the feeds they use and practice responsible antibiotic use when necessary. Fish feed producers should also adhere to standards for antibiotics in fish feeds to protect the safety of the food chain and minimize antimicrobial residues. Responsible use of antibiotics in aquaculture under veterinary supervision is crucial to ensure the safety of aquaculture products. Additionally, education and training should be provided to fish farmers on the proper use of antibiotics, including dosage and withdrawal periods, to reduce the risk of antibiotic resistance. Research on novel feed additives, such as the inclusion of probiotics, prebiotics and medicinal plants in fish feeds, should be conducted to reduce feed costs, improve digestibility, and prevent residual effects of antibiotics in fish tissues that may impact human health.

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Ethical Approval: Not Applicable.

Conflict of Interest: The study was carried out without any conflict of interest.

AUTHORS' CONTRIBUTIONS

OTF conducted the fieldwork and sample collection, in various fish farm locations in Awka, Awka South Anambra State and prepared the initial manuscript. OTF and NCG performed the laboratory procedures, data analysis and interpretation. ICF designed the study, supervised the research and corrected the initial manuscript. All authors reviewed and approved the manuscript for submission.

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