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Abstract
This paper reports on the determination and distribution of antibiotic drugs in poultry feeds mainly the layers and growers mash obtained from Ogun state in Nigeria. Pulverized feed samples were initially treated with phosphate buffer adjusted to pH 7 and the analytes extracted in an SPE cartridge with ammonium hydroxide and methanol. The extract reconstituted in phosphate buffer was determined with high-performance liquid chromatography-diode array detection (HPLC–DAD). The analytical column was an XTerra MS C18 column, (4.6 mm x 100 mm, 3.5 µm) with mobile phases consisting of ultrapure water and acetonitrile mixed with 0.1% HCOOH in gradient elution mode. Data acquisition was achieved with AgilentChemStation Version B.040.01 SP1 while the analytes were completely separated under 10 minutes with good resolution and symmetric peaks. The high correlation coefficient (R^2) values (> 0.998, excluding sulfadimethoxine) indicate a good correlation between analyte concentration and peak areas. Limit of detection (LOD) and quantitation (LOQ) was between 5.37 and 55.42 ng/g, and 17.91 and 184.74 ng/g, respectively. All the drugs exhibited high mean concentration values in the two feed types, and there was no significant difference between their means ($p < 0.05$). The results clearly showed that feed millers fortify their feeds with antibiotics mainly sulfonamides in varying amounts without declaring same, thus compromising security of poultry birds and human consumers.

Keywords: Antibiotic residues, poultry feeds, Food contamination, HPLC-DAD

1. Introduction

Antibiotics as feed additives were globally used to prevent and control many diseases in food-producing animals [1]. Authorization of the majority of these compounds was withdrawn in recent years by several countries including Denmark, Sweden, United Kingdom, The Netherlands and other European Union countries while some went further and banned the inclusion of all essential

antibiotics in feeds as prophylactic agents. These actions arose due to the spread of antibiotic-resistant strains of disease and non-disease causing bacteria commonly found in our ecosystem, and their subsequent transfer through the food chain to humans. This portends severe public health concerns and ecological risks [2]. Meanwhile, a link has been established between excessive usage of antibiotics and antimicrobial resistance [3]. The application of antibiotic drugs in feeds as growth promoters (low doses) far outweighs their uses for therapeutic purposes [4], while animal production consumes about 60% of the total global antibiotic output. In Nigeria, there are no reports on the usage of antibiotics in poultry production. Antibiotic resistant bacteria including Enterococcus faecium resistant to ampicillin, gentamicin, streptomycin, and vancomycin were isolated in tested poultry feeds from the USA, while in another study, poultry feeds represented 85% of all feeds sampled containing bacteria resistant to one or a combination of amoxicillin, cephalothin, clavulanic acid and ampicillin [5]; demonstrating that resistant bacteria in feeds could be transmitted to consuming animals and eventually humans since bacitracin methylene salicylate and virginiamycin applied as growth enhancers in poultry have been reported to alter the chicken intestinal metabolome [6]. Experiments suggested that the use of antibiotics might concomitantly be connected with the development of drug-resistance through a disruption in the natural conditions of the intestine, as indicated in several studies [1].

Antibiotics applications and overuse have been associated with several human health risks. To the best of our knowledge, research findings and literatures about antibiotics occurrence in poultry feedstuffs have not been documented in Nigeria. The aim of this work therefore was to determine enrofloxacin (fluoroquinolone), sulfadimethoxine, sulfamerazine, sulfamethoxazole, sulfamoxole (sulfonamides) and tylosin (macrolide) in non-medicated poultry feed samples using high performance liquid chromatography coupled with diode array detection (HPLC-DAD) after analytical extraction of feed samples in accordance with a previously validated method [3]. This study provides important information regarding the occurrence of antibiotic residues in feedstuffs in order to guarantee the safety of animal products and consequently protect the health of consumers.

2. Methodology

2.1 Chemicals and Reagents

All antibiotic standards and solvents including methanol (HPLC grade, ≥99.9%), NaH_2PO_4, $Na_2HPO_4.12H_2O$, AR ammonium hydroxide were from Sigma-Aldrich (St. Louis, USA), and Deionized Water (Ultrapure water) was produced by an Integral 10 Elix Milli-Q system with an LC (BioPak) polisher (Massachusetts, USA).

2.2 Apparatus

HPLC System - Agilent 1200 series (Agilent Technologies, Germany), HPLC column - XTerra MS C_{18} column, 125Å (4.6 mm x 100 mm, 3.5 μm, particle size) Waters Corporation (Milford Massachusetts, USA) was maintained at 40°C with a column oven, Software - AgilentChemStation Version B.040.01 SP1 Agilent Technologies (Germany), Sieve - Fisherbrand™ 2 mm and Laboratory Miller – RETSCH MM 400 were from Fischer Scientific (New Hampshire, USA), Vortex mixer - Vortexed V-32, Centrifuge - Waring laboratory blender and Nitrogen evaporator - 6 Position N-Evap® were from Thomas Scientific (Swedesboro, USA), Centrifuge tubes - 15 mL and 50 mL (Corning Inc., Corning, NY), SPE Column - SPE (Supelclean™) and Acrodisc®

syringe filters (0.45 μm) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Micropipettes - 1.0 mL and 100 μL were from Eppendorf Co. Ltd. (Hamburg, Germany).

2.3 Standards

500 μg/mL standard solutions of the different antibiotics were prepared by accurately weighing and dissolving 5 mg standard in 5 mL methanol: water (1:1 v/v) and preserved at 4ºC. Working solutions were obtained from the stock by appropriate dilution at time of use. Reagents, standards, and HPLC mobile phase were prepared with ultra-pure water. The LC mobile phase and purified sample extracts were filtered with a 0.45 μm prior to injection into the HPLC system.

2.4 Collection of Feed Samples

Non-medicated poultry feed samples including growers and layers mash totaling 120 were randomly obtained from a list of retail outlets and major poultry farms in different locations in Ogun State, Nigeria. The distribution of samples from the outlets was not equal in number since the number of samples obtained per location depending on the availability and willingness of farmers. The samples on arrival in the laboratory were pulverized and sieved then stored in propylene bottles in the refrigerator. 2 g of pulverized sample was placed into a centrifuge tube and then mixed 10 mL phosphate buffer adjusted to pH 7. The tube was left to stand for 15 mins and vortexed mixed for 10 s at 3500 rpm. The supernatant was transferred to another flask, and the extraction repeated two further times. The extracts were purified using solid phase extraction (SPE) column previously rinsed with 2 mL methanol and ultra-pure water, respectively. The extract upon loading unto the column was washed with 3 mL water/methanol (4:1, v/v). The analytes were eluted with 2 mL 10% ammonium hydroxide/methanol (1:19, v/v). The eluate collected was dried under a mild stream of N_2 at 40ºC. The dried extract was re-dissolved in 1 mL phosphate buffer and cleaned through a 0.45 μm filter and subjected to HPLC analysis.

2.5 HPLC Analysis Experimental Conditions

The HPLC analysis was performed on an Agilent 1200 series under the following operating conditions:
Analytical column: XTerra MS C_{18} column, 125Å (4.6 mm x 100 mm, 3.5 μm, particle size)
Column temperature: 40°C
Mobile phase A: Ultrapure water
Mobile phase B: Acetonitrile
Flow: 1.2 $mlmin^{-1}$
Injection volume: 10 μL
Detector: DAD, 275 nm
Peak width: > 0.10 min (2.5H)
Draw and eject speed: 200 μL /min
Pressure: 600 bar
Post time: 1 min
Data acquisition: AgilentChemStation Version B.040.01 SP1.

2.6 Gradient elution programme for the analysis

Time (min)	Eluent A (%)	Eluent B (%)
0	95	5
6	70	30
12	30	70

2.7 Limits of Detection, Quantification and Analyte Quantification

The retention time for six different antibiotics standards including sulfamozole, sulfamerazine, sulfadimethoxine, enrofloxacin, tylosin and sulfamethoxazole were determined under optimised conditions. Mixed standards of the antibiotics were after that completely resolved over 12 minutes, and calibration curves were constructed using standard antibiotic peak area ratios against retention time (minutes). The curves were constructed over concentration levels of 0 – 5.00 μg/g for each standard from where the analyte concentration in spiked samples was estimated. The limit of detection (LOD) and limit of quantification (LOQ) for each standard were determined from the calibration curve and its linear regression equation assuming that the instrument response y, corresponds linearly to the standard concentration using the model:

$$y = bx + a \quad \text{(i)}$$

which, can be used to express the sensitivity b and LOD and LOQ. LOD and LOQ can, therefore, be expressed as

$$\text{LOD} = \frac{3S_a}{b} \quad \text{(ii)}$$

$$\text{LOQ} = \frac{10S_a}{b} \quad \text{(iii)}$$

where, s_a is the standard deviation (SD) of the response that can be estimated by the standard deviation of either y residuals or y-intercepts of the regression lines and b is the slope of the calibration curve. A 10-point calibration curve was prepared using the standard's retention time and the integrated peak area of the chromatograms to obtain the linear equations are as shown in Table 1.

Table 1: Calibration results for six antibiotic standards

Antibiotics	Linear equation	R^2	LOD	LOQ	y-Absolute	% y-intercept
Sulfamozole	y = 0.0178x + 0.4708	0.9997	5.78	19.27	0.467±0.043	99.97
Sulfamerazine	y = 0.0244x +0.1471	0.9997	5.37	17.91	0.145±0.044	100.08
Sulfadimethoxine	y = 0.0019x + 0.4875	0.9625	55.42	184.74	0.460±0.035	99.9
Sulfamethoxazole	y = 0.0183x + 0.5757	0.9996	8.46	28.20	0.613±0.052	99.96
Enrofloxacin	y = 0.0200x + 0.8996	0.9997	9.23	30.75	0.857±0.062	99.95
Tylosin	y = 0.0039x + 0.1864	0.9993	8.385	27.95	0.183±0.011	100.22

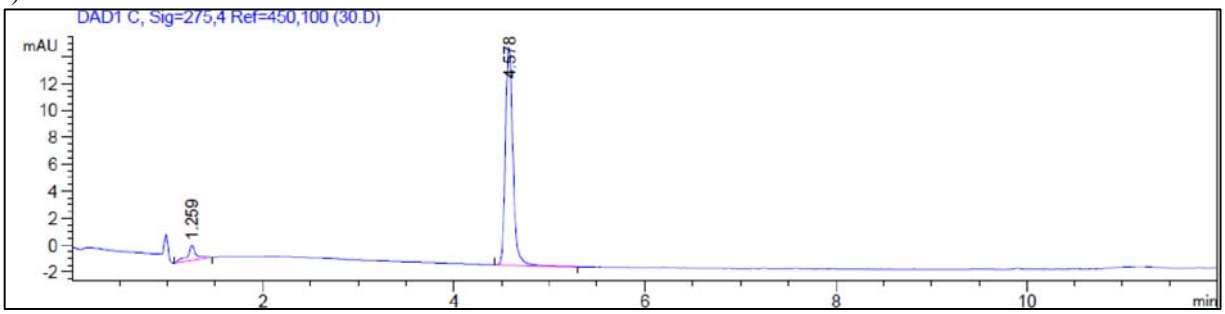
Linearity and regression study was done at λ_{275} for each antibiotic. The high correlation coefficient (R^2) values (all greater than 0.998, excluding sulfadimethoxine) indicate a good correlation between analyte concentration and peak areas.

3. Results and discussion

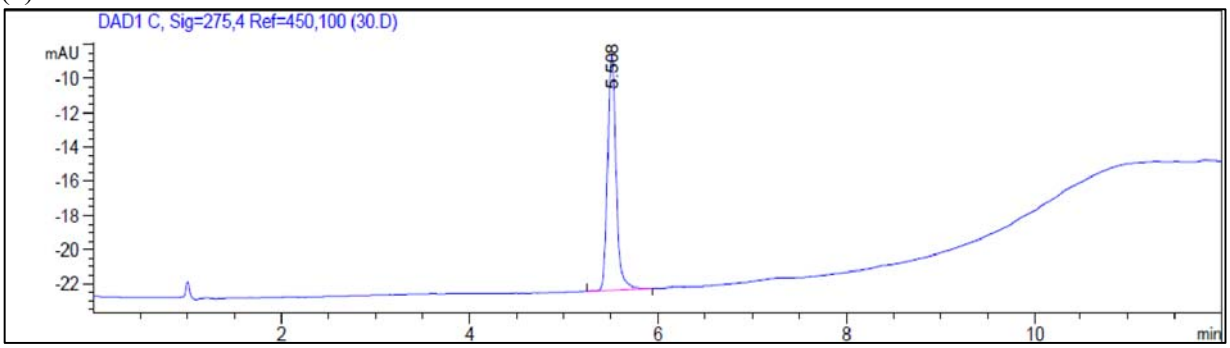
3.1 Order of Elution of Standard Antibiotics

The antibiotics were eluted singly from the column after optimising the chromatographic parameters, and their retention time obtained (Figure 1). A mixed standard of the different antibiotics was after that prepared with a concentration range of 0 – 500 µg/kg. The six antibiotics including Sulfamoxole (SMX), sulfamerazine (SMR), sulfadimethoxine (SDM), enrofloxacin (ENR), tylosin (TYL) and sulfamethoxazole (SMZ) in that order were eluted as shown in Figure 2. Analytes were quantified using their peak areas as obtained in the chromatograms (Figure 3) from linear equations.

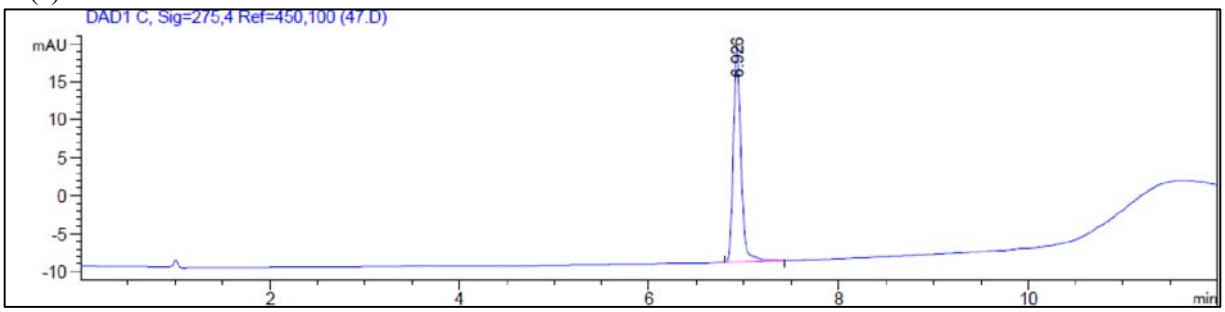
(a)



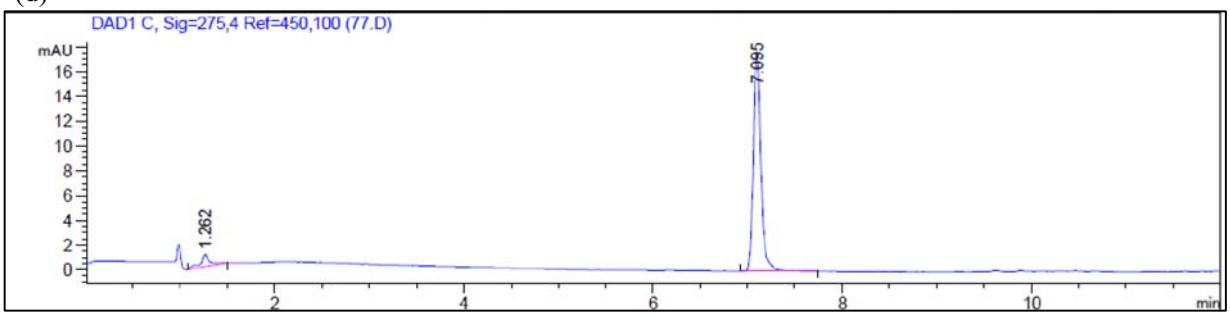
(b)



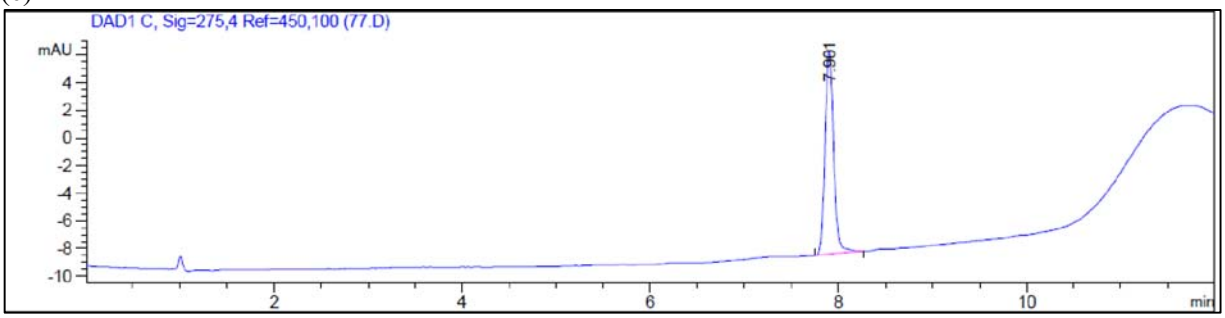
(c)



(d)



(e)



(f)

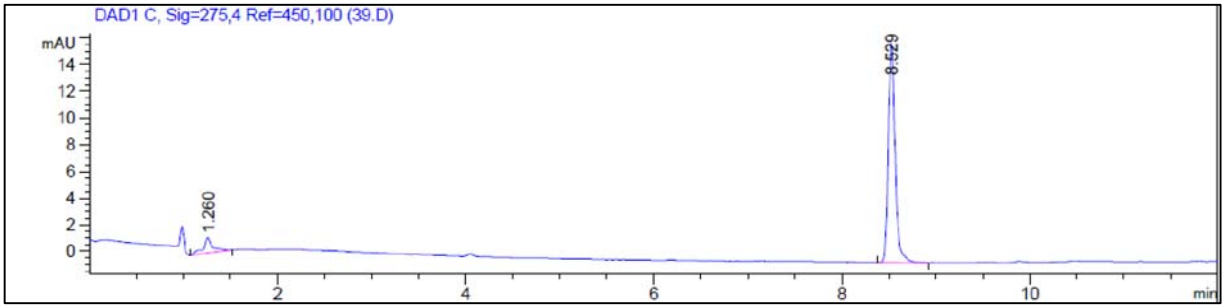


Figure 1: Chromatograms of the six antibiotic standards: (a)SMX, (b)SMR, (c)SDM, (d)ENR, (e)TYL, (f)SMZ

There were no interfering peaks in the blank chromatograms at the quantification wavelength (275 nm). As shown in Fig. 2, the peaks of the 6 antibiotics show that the separation of all the compounds was successfully completed in less than 10 min, with good resolution and symmetric peaks.

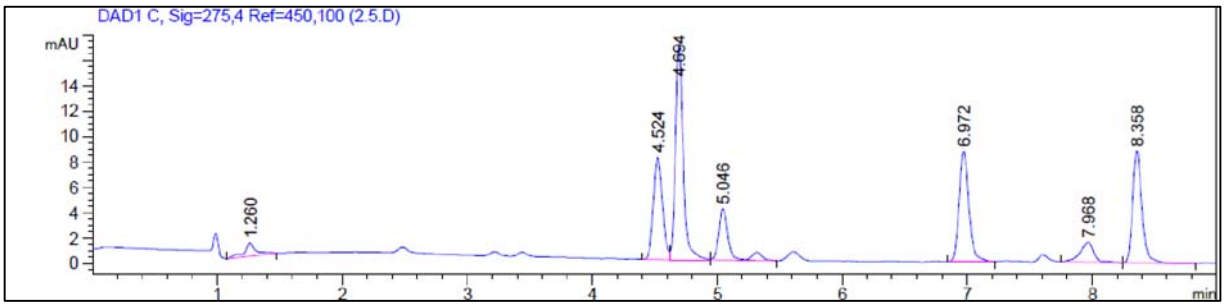


Figure 2: HPLC chromatogram of standard mixture at 275 nm: (1)SMX, (2)SMR, (3)SDM, (4)ENR, (5)TYL, (6)SMZ

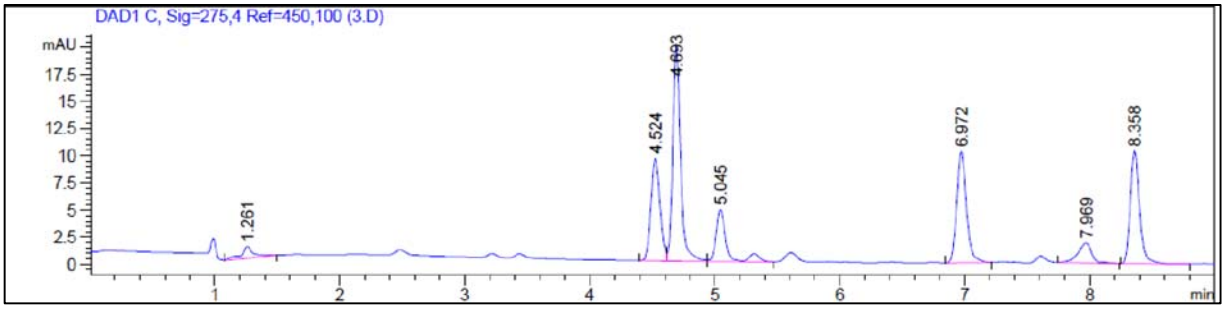


Figure 3: Typical HPLC chromatogram obtained from the feeds

3.2 Distribution of Antibiotics Drugs in Poultry Feeds

Enrofloxacin, sulfadimethoxine, sulfamerazine, sulfamethoxazole, sulfamoxole and tylosin were determined in poultry feeds consisting of growers' and layers mash as indicated in Table 4.5. Enrofloxacin occurs at a mean concentration of 526.71±231.83 and 295.75±98.97 ng/g in growers and layers mash, respectively at 85.71% and 77.75% for the 2 feed types. Sulfadimethoxine was in 57.14% of the growers' mash but with a mean concentration of 52065.80±13620.16 ng/g with a maximum concentration of 90907.90 ng/g. This scenario was however not the same in the layers mash for sulfadimethoxine as the sample had a higher occurrence of the drug (66.67%) and also with a mean of 13399.12±9039.72 ng/g. The maximum concentration of sulfadimethoxine that was in the layers mash was lower compared with that obtained for the growers' mash. Sulfamerazine was at an average concentration of 420.73±111.77 and 328.11±126.68 ng/g for the growers' and layers mash, respectively and in 71.43 and 66.67% of the feeds. Sulfamethoxazole was in more numbers of growers mash at 66.67% occurrence and with an average concentration of 214.60±126.62 ng/g and a maximum concentration of 567.06 ng/g. 181.80±102.27 ng/g was the mean concentration for sulfamethoxazole in the layers mash, and it was present in 44.44% of the samples with a maximum concentration of 497.62 ng/g.

Growers and layers mash had 17.48±1.36 and 16.35±1.55 ng/g sulfamoxole, respectively. The drug occurred in 75.43% and 66.67% of the feeds with maximum concentrations of 21.5 and 22.8 ng/g for the growers and layers mash in that order. Tylosin was in both feed types at 42.86% and

33.33% at an average concentration of 579.63±445.43 and 442.87±207.25 ng/g for the growers and the layers mash, respectively. The maximum concentrations for the two feeds were 1468.52 and 699.28 ng/g for the growers and layers mash, respectively. Enrofloxacin, sulfamerazine, tylosin, and sulfamethoxazole in that order were the main antibiotics in the grower mash while sulfadimethoxine, enrofloxacin, sulfamerazine and tylosin were the most common antibiotics in the layers mash. Enrofloxacin approved for the treatment of poultry against *Escheridia coli* and *Pasteurella multocida* was significantly present in the feeds under consideration in this study. Accumulation of enrofloxacin residue in foods could lead to treatment failures in respect of severe gastrointestinal infections triggered by *Salmonella* in humans, and resistance to this antibiotic has been documented. A five-day withdrawal period is recommended for chickens treated with enrofloxacin before slaughter [7], a recommendation mostly ignored by farmers.

Kim et al. (2016) [8] had reported SAs in animal feeds from the Republic of Korea with sulfamerazine found in all of the samples analysed been the most used SA in animal feeds. The observation of Kim et al. is consistent with the results obtained in the present with respect to sulfonamides, however, sulfadimethoxine was the most prevalent SA among the different feeds.
The accumulation of antibiotics in foods lead to risks including but not limited to selection pressure that causes bacterial resistance, aplasia of the bone marrow, changes in the bacterial flora, tumor induction and hypersensitivity reactions [9]. Compounds including tylosin, β-lactams and systemic sulfonamides detected in the present study have been banned for use as growth promoters by countries such as Brazil and other EU member countries [9].

However, sulfonamides are widely used as growth promoters since they are relatively inexpensive and the short-life sulfonamides including sulfadiazine, sulfamethoxazole and sulfamoxole are mixed with feeds to prevent bacterial infection [10], thus suggesting their high mean concentration in the feeds examined in this study. Residues of sulfonamides in foods have the capacity to cause a range of untoward reactions such as haemopoietic and urinary tract disorders, hypersensitivity and porphyria reactions that could result in severe cutaneous adverse reactions [11]. Other consequences associated with high ingestion of sulfonamides may include but not limited to conditions including glossitis, granulomatous hepatitis, stomatitis, and diffuse macular or vesicular rashes [12].

Table 2: Antibiotic Residues in Poultry Feeds (n = 60) in ng/g

	GM			LM		
Antibiotics	Mean±SD	%	Max	Mean±SD	%	Max
Enrofloxacin	526.71[a]±231.83	85.71	1500.04	295.75[a]±98.97	77.78	690.04
Sulfadimethoxine	52065.80[a]±13620.16	57.14	90907.90	13399.12[a]±9039.72	66.67	57855.26
Sulfamerazine	420.73[a]±111.77	71.43	750.24	328.11[a]±126.68	66.67	856.8
Sulfamethoxazole	214.60 [a]±126.62	66.67	567.06	181.80[a]±102.27	44.44	479.62
Sulfamoxole	17.48[a]±1.36	71.43	21.5	16.3 [a]±1.55	66.67	22.8
Tylosin	579.63[a]±445.43	42.86	1468.52	442.87[a]±207.25	33.33	699.28

Key: GM – Growers' mash; LM – Layers mash; % - Percent of samples containing each drug;
[a]Means across the same row are not significantly different ($p < 0.05$)

4. Conclusion

Results from this study revealed the presence of fluoroquinolone, sulfonamide and macrolide deliberately included as food additives but not declared on the labels of these products thereby exposing consuming animals and the public to antibiotic resistance arising from the presence of residues in the different of poultry birds and eggs. The sulfonamides were the preponderant drugs encountered because they have been reported as additives added to feeds to combat bacterial growth. An improvement is therefore required in the surveillance capabilities of key monitoring agencies to curb excessive use of antibiotics, and confiscation of food items with excess drug loads. An enlightenment campaign is required to educate feed producers, and farmers alike on the dangers inherent in the inclusion of antibiotics in their products.

5. Recommendation

Effective alternatives to antibiotics in poultry production are probiotics, and other means to reduce pathogens. Probiotics are feed additives fortified with pure or mixed cultures of live microorganisms that stimulate beneficial effects in the host by favoring intestinal microbiota balance, though their modes of action is not fully understood yet. Exogenous enzymes and plant extracts are also added to improve their digestibility and nutrients release, while the essential oils of plant extracts assist in poultry performance by exerting antimicrobial and antifungal effects, stimulate the excretion of endogenous enzymes, change the intestinal microflora, and ultimately help to reduce subclinical infections. Natural growth promoters exclusively of botanical origin including essential oils and phytochemicals are currently been exploited as alternatives to antibiotic growth promoters in poultry production. These alternatives improve the health of poultry against various infectious diseases rather than regular nutrition. Vaccination with non-pathogenic vaccines is been increasingly used in commercial poultry farms, instead of in-feed antibiotics.

Acknowledgements

The authors wish to acknowledge the financial support of the Tertiary Education Trust Fund (Tetfund) of Nigeria and Covenant University.

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