



Growth Performance and Gut Morphological Responses of Broiler Chickens to Fermented Diets

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Authors' contributions

This work was carried out in collaboration among all authors. Author OAO designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author CCO managed the data collection procedure and analyses of the study. Author AMO managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Growth performance and gut morphological response of broiler chickens fed moist fermented diets with the inclusion of probiotics or/and organic acids were evaluated in a 56 day feeding trial.

Place and Duration of Study: Poultry unit of the Department of Agricultural Education, Federal College of Education, Abeokuta, Ogun State, Nigeria between February 2018 and April 2018

Methodology: Three hundred one-day old unsexed Abor Acre broilers were used for this study. They were randomly allotted to 5 dietary treatments with 6 replicates of 10 birds each. The experimental treatments were: Diet 1: Dry Unfermented Feed (DUF) - Probiotics (Pr) – Organic acids (Or), Diet 2: Moist Fermented Feed (MFF) – Pr – Or, Diet 3: MFF + Pr, Diet 4: MFF + Or, Diet 5: MFF + Pr + Or. The experiment was carried out using a completely randomized design.

Results: The feed conversion ratio of broiler starters fed diet 5 (1.96) was significantly ($P < .0001$) better relative to those of birds fed other diets (2.27, 2.21, 2.14 and 2.13 respectively), while birds in treatment 1 (2.27) had significantly ($P < .0001$) poor feed conversion ratio. There were no significant ($P > .05$) differences in the feed conversion ratio of broiler finishers fed MFF with or without feed additives (2.68, 2.64, 2.55 and 2.55). However, the value of feed conversion ratio of birds fed DUF (3.04) was significantly ($P = .05$) the highest. The duodenal villus height to crypt depth

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ratio of broiler starters fed MFF+ Pr + Or (7.11) was significantly ($P=0.05$) higher than those of birds fed other diets (2.70, 3.35, 3.98, 4.73 respectively).

Conclusion: Although, feed fermentation enhanced gut morphological parameters which correlates improved growth performance of broiler chickens. The inclusion of probiotics (1g/kg) and organic acids (1g/kg) in the fermentation process further improved the growth indicators of broiler chickens used in this study.

Keywords: Performance; feed fermentation; probiotics; organic acids; gut morphology.

1. INTRODUCTION

The poultry industry is one of the fastest growing meat and egg producing sector. Therefore, better feed efficiency and good performance of the birds are the crucial goals in poultry production, in order to bridge the gap between demand and supply of animal protein which exists in Nigeria. Feed cost accounts for more than 60% of total poultry production, hence strategies should be deployed to improve feed efficiency.

In the past, using antibiotics to promote the growth of poultry and manage gut microbiota was a norm. The general mode of action of antibiotics as growth promoter are: inhibition of sub-clinical infections, reduction of growth-depressing microbial metabolites, reduction of microbial use of nutrients and enhanced uptake and use of nutrients through the thinner intestinal wall [1-3]. However, due to concerns over potential fatalistic impacts on food animals and indirectly to humans, their use as feed additives are banned or regulated in several jurisdictions. In this changed context, several alternative strategies have been proposed with some success that mimics the functions of antibiotics as growth promoters and modulate gut microbiota for their beneficial roles. These include the use of probiotics, prebiotics, organic acids etc. Gut microbiota and their metabolic products improve nutrient digestion, absorption, metabolism, and overall health and growth performance of poultry.

Fermentation has been used to improve the quality of food for a long time. It is described as the conversion of organic chemicals into simpler compounds using active enzymes or complex organic catalysts produced by microorganisms such as bacteria, yeasts, or moulds. It has been utilized to improve the nutritional value of a variety of feed materials, including soybean [4] and copra meal [5]. Besides from improved nutritional properties, fermentation is characterized by increasing the number of lactic acid bacteria, reducing pH and increasing the concentration of organic acids in a product [6].

These features may protect feed from pathogen contamination [7], promote chicken gastrointestinal health [8], as well as chicken growth and development [9,10].

Feeding fermented diet to pigs has been a widespread practice for many years [6], but there is currently a growing interest in adding fermented feed into broiler rations to benefit from its positive effects, particularly on gut health and production characteristics [11]. It promotes the growth of microorganisms that break down fiber and anti-nutrients, alters the bacterial ecology of the gastrointestinal tract, and lowers the number of *Enterobacteriaceae* in various sections of the gastrointestinal tract, as shown in pigs [12] and broiler chicks [13].

The acidic conditions of the fermented feed result in a decrease pH of the gastrointestinal tract which in turns increases the number of microorganisms responsible for lytic action in the gastrointestinal tract resulting in lower digesta passage rate, thus, increasing the amount of time the feed is exposed to enzymatic action which leads to enhanced digestibility.

Typically commercial poultry birds are fed dry compounded feed but the feeding of wet feed has been reported to significantly beneficial [14]. Through many mechanisms of action, fermented wet feed improves the health of poultry by enhancing the environment of the gastrointestinal tract, resulting in improved chicken performance.

Moist fermented feed is a microbial product that was first proposed for improving various feed components by allowing beneficial microorganisms (especially cellulolytic bacteria) to pre-digest them, resulting in digested feed that increases the availability of some nutrients to the host.

Probiotics are live microbial feed supplements that benefits the host animal by enhancing the microbial balance in its intestine. *Lactobacillus*, *Saccharomyces*, *Streptococcus*, *Bacillus*,

Lactobacillus, *Saccharomyces* and *Aspergillus* are the most common probiotic strains [15-17]. The ability of *Lactobacillus* to breakdown carbohydrate to form lactic acid is crucial in feed fermentation.

Organic acids are effective in chicken not only as a growth promoter, but also as a strategy for managing all pathogenic and non-pathogenic intestinal bacteria [18,19]. Furthermore, organic acids feeding is thought to have a number of positive impacts, including boosting feed conversion ratios, growth performance and mineral absorption [20-23]. Organic acids, unlike antibiotics, have additional effects like as decreasing the gastric pH and thereby improving protein digestion [24]. Acidification is a means of controlling the growth of undesirable microorganisms including pathogens.

Since very few investigations on the spontaneous fermentation of broiler chickens feed with or without additives (probiotic and organic acid) have been done, therefore, this study evaluated the comparative effects of spontaneously fermented feed and feed fermented with additives (probiotic or/and organic acids) on the gut morphology and broiler performance.

2. MATERIALS AND METHODS

The study was carried out at the Department of Agricultural Education, Federal College of Education, Abeokuta, Ogun State, Nigeria. A total of three hundred one-day old Abor acre broiler chicks were used for this study. They were randomly allotted to five dietary treatments which was replicated six times with ten birds in each replicate group. The birds were housed in a deep litter floored poultry pen with twenty hours access to light.

The chicks were brooded in groups for a period of seven days, and the brooding temperature was maintained close to the standard broiler guide requirements. The litter bedding was made up of dry wood shavings and a high level of hygiene was maintained throughout the experimental period to ensure an environment conducive for growth.

Compounded complete broiler starter and finisher feed were purchased from a reputable feed company. The chicks were fed starter diet containing 2800 KcalME/Kg, 22%CP for four weeks (1-28 days) after which they were fed

finisher diet containing 3000 KcalME/Kg, 20%CP for four weeks (28-56 days). Feed and water were supplied *ad-libitum*.

Complete starter and finisher feed were fermented with and without the addition of probiotic or organic acid or their combination. The feed was moistened with water (1 liter water: 1 kg feed), placed in a plastic bucket. Moist fermented feed with additives were prepared using either 2g of probiotics per kilogram of feed or 2g of organic acid per kilogram of feed or a combination of 1g of probiotics and 1g of organic acid/ kg of feed. Then, the plastic buckets were kept and incubated in a small room for 48 h. at 37±2°C. The probiotic product (LACTOPLUS) which is a blend of the following selected probiotic strains *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus casei*, *Lactobacillus reuteri*, *Lactobacillus lactis*, *Streptococcus faecium*, *Aspergillus oryzae* and *Saccharomyces cerevisiae* was used for the research. While the constituents of the dietary acidifier (DIGECID) used for this study are Formic, Fumaric, Citric and Lactic acids.

The dietary treatments are as follows:

- T1: Dry Unfermented Feed (DUF) without probiotics or organic acids
- T2: Moist Fermented Feed (MFF) without probiotics or organic acids
- T3: Moist Fermented Feed (MFF) with probiotics (2 g/kg of feed)
- T4: Moist Fermented Feed (MFF) with organic acids (2 g/kg of feed)
- T5: Moist Fermented Feed (MFF) with probiotics or organic acids (1g/kg of feed each)

2.1 Growth Performance

The body weight and feed consumption were monitored and recorded for each replicate weekly in order to determine the average feed intake, average body weight gain and feed conversion ratio of the birds. The birds were weighed on the first day of the experiment, then weighed weekly throughout the remaining experimental period. Feed was given daily and the leftover feed was weighed weekly to calculate the feed conversion data with equations as shown below:

$$\text{Live weight/bird} = \frac{\text{Total weight of birds (g)}}{\text{Number of birds weighed}}$$

$$\text{Body weight gain} = \text{final body weight} - \text{initial body weight}$$

Feed Intake (FI) = Feed given – Feed left over.

$$\text{Feed conversion ratio (FCR)} = \frac{\text{Total feed consumed (g)}}{\text{Weight gained (g)}}$$

2.2 Gut Morphology

At the end of the 4th and 8th week, a bird per replicate was selected for morphological analysis. One bird per replicate was sacrificed using an approved method, to collect the small intestine. The gastrointestinal tract was removed and separated into the three intestinal segments (duodenum, jejunum and ileum). The duodenum was taken distally from the gizzard to the end of the pancreatic loop, the jejunum was taken distally from the pancreatic loop to Meckel's diverticulum, and the ileum was taken from the Meckel's diverticulum to the ileo-caecal junction. A sample portion of 0.5 cm in length was taken from the middle portion of each of the three segments (Duodenum, Jejunum and Ileum) collected and placed into separate sample bottles containing 10% formalin for histological measurements. The samples were kept for 24 h in acetic acid/ethanol (25/75, v/v), after which it was rehydrated in a bath of ethanol/water (50/50, v/v) and then in distilled water. Thereafter, the samples were stained with the Feulgen reaction: hydrolysed in 1 N HCL at 60 °C for 6 min, it was rinsed three times with distilled water and stained with Schiff reagent for 30 min. The samples were rinsed in distilled water and stored in acetic acid/water (45/55, v/v) at 4 °C until further analysis. Intestinal villi with their crypts were separated individually under a dissecting microscope as described by [25]. The length and width of the villi were measured according to the procedure described by [26] using an optical microscope and a camera.

2.3 Statistical Analysis

All data obtained from the study were subjected to Analysis of Variance using GLM procedures within SAS software [27]. Statistically different means were separated using Duncan's Multiple Range Test ($P < .05$).

3. RESULTS AND DISCUSSION

3.1 Growth Performance

Growth performance of broiler chickens fed with experimental diets at the starter and finisher phases are shown in Tables 1 and 2 respectively. Feed fermentation significantly ($P = .05$) had positive influence on the body

weight gain of the experimental birds at the starter phase (Table 1). Birds fed diet 5 had the highest ($P = .05$) body weight gain (795.72g), followed by birds fed diets 4, 3, 2 and 1 (748.71g, 747.13g, 746.42g and 721.78g respectively). The body weight gain of birds fed DUF without feed additives was significantly the lowest. It was observed at both weeks 4 and 8 that the administration MFF to broiler starters significantly ($P = .05$) reduced their feed conversion ratio hence improving the feed efficiency of the birds. However, birds fed MFF with the inclusion of either probiotics or organic acids or their combination significantly utilized feed consumed more efficiently.

The results of this study suggests that the improvement of body weight gain might be associated with the ability of beneficial microorganisms to secrete enzymes such as amylase, protease and lipase which might improve the digestion rate of feed nutrients. This connotes that feed fermentation promotes the growth of rich microorganism that can assist the digestion of feed as reported by [12].

The values obtained in this research can be explained by the ability of organic acids to reduce pH, accelerates the conversion of pepsinogen to pepsin, which improves the absorption rate of proteins, amino acids, and trace minerals [28]. Additionally, organic acids improves energy digestibility by reducing the microbial competition for nutrients in the host. These are similar to previous reports that the addition of organic acids to broiler diets improved weight gain [29,30] and improved feed conversion ratio [31].

This result can also be strongly supported when high feed conversion ratio value was reported for the control group compared to the groups of birds fed probiotics based on *Lactobaccillus* sp. and *Saccharomyces cerevisiae* in their diets [32-34]. Probiotics prevent such harmful bacteria (enteric pathogens) from growing in the gut and thus minimize the disturbances caused by them, and also maintain host favorable bacteria.

3.2 Gut Morphology

The gut morphological indices of the experimental birds at starter and finisher phases are presented in Tables 3 and 4. The trend observed in gut morphology at 4 and 8 weeks of age of broiler chickens showed that duodenal, jejunal and ileal villus height increased significantly ($P = .05$) with moist fermented feed across the experimental treatments.

These results in Tables 3 and 4 are similar to those reported by [35] and [36]. It also corroborated the trend observed for growth performance in this study, this might help to explain the improvements in body weight gain and feed efficiency. This is in agreement with [23] who reported that feed acidification positively influences the gut morphological parameters of broiler chickens.

The intestine, especially the crypts and villi surface area of the absorptive epithelium, play significant roles in the final stages of nutrient digestion and assimilation [37]. The crypt is the production site where divisions of stem cells occur to allow villus renewal. Hence, a deeper crypt suggests a fast cellular turnover to permit villus renewal which is needed in response to inflammation resulting from the effects of pathogens [38].

Table 1. Growth performance of broiler starters fed experimental diets

| Treatment | 1 | 2 | 3 | 4 | 5 | P-value | SEM |
|-----------|----------------------|----------------------|-----------------------|-----------------------|----------------------|---------|-------|
| IW | 40.22 | 40.24 | 40.20 | 40.29 | 40.28 | 0.8009 | 0.05 |
| FW | 762.00 ^b | 786.67 ^b | 787.33 ^b | 789.00 ^b | 836.00 ^a | <0.0001 | 3.15 |
| BWG | 721.78 ^c | 746.42 ^b | 747.13 ^b | 748.71 ^b | 795.72 ^a | 0.0057 | 3.15 |
| FI | 1637.07 ^a | 1646.67 ^a | 1595.67 ^{ab} | 1596.00 ^{ab} | 1558.33 ^b | <0.0001 | 11.71 |
| FCR | 2.27 ^a | 2.21 ^{ab} | 2.14 ^b | 2.13 ^b | 1.96 ^c | <0.0001 | 0.02 |

IW-Initial Weight; FW-Final Weight; BWG- Body Weight Gain; FI- Feed Intake and FCR-Feed Conversion Ratio. Means on the same row with different superscripts are significantly (P<0.05) different

Table 2. Growth performance of broiler finishers fed experimental diets

| Treatment | 1 | 2 | 3 | 4 | 5 | P-value | SEM |
|-----------|----------------------|-----------------------|-----------------------|-----------------------|----------------------|---------|-------|
| IW | 762.00 ^c | 786.67 ^b | 787.33 ^b | 789.00 ^b | 836.00 ^a | <0.0001 | 3.15 |
| FW | 1701.00 ^b | 1756.00 ^{ab} | 1796.33 ^{ab} | 1804.67 ^{ab} | 1846.67 ^a | 0.0174 | 19.02 |
| BWG | 939.00 | 969.33 | 1009.00 | 1015.67 | 1010.67 | 0.1549 | 16.99 |
| FI | 2846.67 ^a | 2592.67 ^{bc} | 2666.33 ^b | 2592.00 ^{bc} | 2572.67 ^c | <0.0001 | 15.28 |
| FCR | 3.04 ^a | 2.68 ^b | 2.64 ^b | 2.55 ^b | 2.55 ^b | 0.0017 | 0.05 |

IW-Initial Weight; FW-Final Weight; BWG- Body Weight Gain; FI- Feed Intake and FCR-Feed Conversion Ratio. Means on the same row with different superscripts are significantly (P<0.05) different

Table 3, Gastrointestinal morphology of broiler starters fed experimental diets (Week 4)

| Treatment | 1 | 2 | 3 | 4 | 5 | P-value | SEM |
|-----------------|----------------------|----------------------|----------------------|----------------------|----------------------|---------|-------|
| Duodenum | | | | | | | |
| VH | 943.31 ^c | 956.20 ^{bc} | 976.63 ^{bc} | 981.25 ^b | 1182.51 ^a | <0.0001 | 6.60 |
| AW | 31.43 ^d | 34.34 ^{cd} | 39.08 ^{bc} | 42.06 ^{ab} | 44.97 ^a | <0.0001 | 0.96 |
| BW | 83.75 ^b | 155.39 ^a | 162.26 ^a | 162.86 ^a | 164.00 ^a | <0.0001 | 3.43 |
| CD | 349.65 ^a | 285.09 ^b | 246.44 ^c | 208.93 ^d | 166.42 ^e | <0.0001 | 6.58 |
| VH:CD | 2.70 ^d | 3.35 ^{cd} | 3.98 ^{bc} | 4.73 ^b | 7.11 ^a | <0.0001 | 0.13 |
| Jejunum | | | | | | | |
| VH | 752.16 ^{ab} | 728.41 ^b | 812.27 ^{ab} | 803.44 ^{ab} | 850.53 ^a | 0.0199 | 19.01 |
| AW | 39.49 ^c | 44.53 ^c | 44.41 ^c | 53.72 ^b | 61.60 ^a | <0.0001 | 1.08 |
| BW | 90.53 ^b | 98.33 ^{ab} | 93.76 ^{ab} | 107.63 ^{ab} | 110.04 ^a | 0.0196 | 3.52 |
| CD | 190.67 ^a | 178.33 ^{ab} | 160.67 ^{bc} | 155.33 ^c | 150.00 ^c | 0.0006 | 4.45 |
| VH:CD | 3.95 ^b | 4.09 ^b | 5.06 ^a | 5.18 ^a | 5.69 ^a | <0.0001 | 0.14 |
| Ileum | | | | | | | |
| VH | 623.71 ^b | 639.78 ^b | 652.63 ^b | 656.65 ^b | 796.27 ^a | 0.0002 | 12.83 |
| AW | 48.60 ^c | 56.83 ^{bc} | 59.33 ^b | 56.66 ^{bc} | 69.14 ^a | 0.0003 | 1.64 |
| BW | 95.63 | 102.74 | 102.64 | 98.57 | 97.30 | 0.7190 | 3.81 |
| CD | 257.67 ^a | 247.15 ^a | 206.14 ^b | 194.29 ^{bc} | 182.20 ^c | <0.0001 | 3.80 |
| VH:CD | 2.42 ^c | 2.59 ^c | 3.18 ^b | 3.38 ^b | 4.37 ^a | <0.0001 | 0.10 |

VH-Villus Height; AW-Apical Width; BW- Basal Width; CD-Crypt Depth and VH:CD-Villus Height to Crypt Depth Ratio. Means on the same row with different superscripts are significantly (P<0.05) different

Table 4. Gastrointestinal morphology of broiler finishers fed experimental diets (Week 8)

| Treatment | 1 | 2 | 3 | 4 | 5 | P-value | SEM |
|-----------------|---------------------|---------------------|-----------------------|-----------------------|----------------------|---------|-------|
| Duodenum | | | | | | | |
| VH | 977.67 ^c | 994.00 ^c | 1068.67 ^{bc} | 1163.00 ^{ab} | 1217.67 ^a | <0.0001 | 18.44 |
| AW | 42.87 ^c | 45.62 ^{bc} | 47.83 ^b | 48.13 ^b | 54.47 ^a | <0.0001 | 0.58 |
| BW | 78.60 ^c | 82.96 ^{bc} | 85.13 ^{bc} | 90.26 ^b | 101.84 ^a | 0.0003 | 1.80 |
| CD | 157.44 ^a | 152.29 ^a | 141.73 ^b | 127.11 ^c | 124.24 ^c | <0.0001 | 1.77 |
| VH:CD | 6.21 ^c | 6.53 ^c | 7.54 ^b | 9.16 ^a | 9.80 ^a | <0.0001 | 0.19 |
| Jejunum | | | | | | | |
| VH | 472.25 ^c | 484.31 ^c | 677.73 ^b | 745.59 ^b | 920.94 ^a | <0.0001 | 11.83 |
| AW | 64.86 ^c | 75.20 ^b | 86.92 ^a | 89.03 ^a | 87.96 ^a | <0.0001 | 0.67 |
| BW | 114.80 ^c | 134.82 ^b | 141.44 ^{ab} | 154.13 ^a | 154.36 ^a | <0.0001 | 2.31 |
| CD | 244.42 ^a | 225.72 ^b | 214.14 ^{bc} | 204.50 ^c | 143.41 ^d | <0.0001 | 3.41 |
| VH:CD | 1.94 ^d | 2.15 ^d | 3.17 ^c | 3.64 ^b | 6.42 ^a | <0.0001 | 0.07 |
| Ileum | | | | | | | |
| VH | 579.69 ^c | 589.74 ^c | 616.13 ^b | 631.84 ^{ab} | 647.56 ^a | <0.0001 | 3.95 |
| AW | 48.84 ^c | 60.74 ^b | 63.92 ^b | 61.59 ^b | 72.76 ^a | <0.0001 | 0.82 |
| BW | 99.37 ^b | 106.20 ^b | 136.33 ^a | 138.73 ^a | 148.68 ^a | 0.0003 | 4.42 |
| CD | 218.62 ^a | 213.25 ^a | 178.17 ^b | 156.58 ^c | 110.51 ^d | <0.0001 | 1.77 |
| VH:CD | 2.65 ^d | 2.77 ^d | 3.46 ^c | 4.04 ^b | 5.87 ^a | <0.0001 | 0.06 |

VH-Villus Height; AW-Apical Width; BW- Basal Width; CD-Crypt Depth and VH:CD-Villus Height to Crypt Depth Ratio. Means on the same row with different superscripts are significantly ($P<0.05$) different

The significantly ($P=0.05$) higher duodenal, jejunal and ileal villi height obtained in this study with MFF fed birds suggested increased surface area capable of greater absorption of available nutrients [39]. Significantly ($P=0.05$) deeper crypt observed in birds fed DUF without probiotics or organic acids as opposed to the values of crypt depth observed in birds fed MFF with or without feed additives suggests higher pathogenic activities in the birds.

This is in accordance with report by [13] that the utilization of fermented feed in broiler nutrition alters the bacterial ecology of the gastrointestinal tract, and lowers the number of *Enterobacteriaceae* in various sections of the gastrointestinal tract.

Greater villus height is an indication of activation in the function of the intestine leading to higher potential capacity for absorption of nutrients [40]. This study has indicated the existence of a direct relationship between the feeding of diets rich in beneficial microorganisms on villus height, which enhances the intestinal absorptive surface thus leading to a greater potential nutrient absorption [34].

The villus height to crypt depth ratio is a very useful assessment to estimate the absorption capacity of the small intestine. Maximum digestion and absorption are associated to increased villus height to crypt depth ratio [35]. In

this study, the villus height to crypt depth ratio at the different segments of the gastrointestinal tract followed a particular pattern at the starter and finisher phases, where the birds fed MFF with the inclusion of probiotics and organic acids showed a significantly ($P=0.05$) higher value than birds fed other experimental diets. This might be associated with the increased number of beneficial bacteria such as *Lactobacilli*, *Bifidobacterium*, *Bacillus subtilis* and *Saccharomyces cerevisiae* (Naji and Al-Mosawi, 2004). The addition of probiotics or/and organic acids could have led to higher production of secondary metabolites during fermentation. Some of the metabolites that are produced during fermentation include lactic acid from *Lactobacilli*, enzymes like amylase and protease and antimicrobials like iturin and surfactin produced by *Bacillus subtilis* bacteria [41].

4. CONCLUSION

In conclusion, the utilization of feed fermentation as a strategy in broiler nutrition improved the feed efficiency and gastrointestinal morphology of broiler chickens. However, the inclusion of probiotics and organic acids in the fermentation process of feed increased the benefits of feeding moist fermented feed to broiler chickens.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our

area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by Tertiary Education Trust Fund (TETFund), Nigeria.

ETHICAL APPROVAL

The practices adopted in the method of this study were carried out according to the guidelines as approved by the project review committee of the Department of Agricultural Education, Federal College of Education, Abeokuta, Ogun state, Nigeria.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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