Assessment of Caecal Microbiome in two Breeds of Rabbits Fed Fermented Cocoa Podhusk Meal

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Abstract—The study was conducted to determine the effect of inclusion of fermented cocoa pod husk meal in feed of rabbits on the microbial flora in the caecum. Sixty 5 weeks (35 days) old grower rabbits comprising 2 breeds (thirty New Zealand White and thirty Chinchilla) were used for the study. They were randomly distributed into six treatments of 10 rabbits per group and fed diets containing fermented cocoa pod husk meal (FCPHM) at 0%, 12.5% and 25% inclusion levels for a period of 8 weeks. Contents of the caecum of experimental rabbits were collected at the end of the feeding trial for analysis of bacterial counts/load, identification and characterization of the bacteria organisms. The results showed that the different breeds of rabbit and varying inclusion levels of FCPHM had significant influence (P<0.05) on the bacterial and coliform counts in the caecum. The average caecal bacterial and coliform population increased as the inclusion levels of FCPHM increased with rabbit fed control diet having 56.86 × 10² cfu/ml; 7.38 × 10² cfu/ml; those fed 12.5% FCPH having 87.82 × 10² cfu/ml; 10.63 × 10² cfu/ml and those fed 25% FCPHM having 101.63 × 10² cfu/ml; 15.25 × 10² cfu/ml bacteria and coliform counts respectively. The breed effect also showed that New Zealand White had higher bacterial and coliform counts of 93.67 × 10² cfu/ml and 13.58 × 10² cfu/ml when compared to Chinchilla with 70.58 × 10² cfu/ml and 8.56 × 10² cfu/ml. Only Salmonella and Bacillus species of bacteria were commonly isolated from the rabbits fed the three dietary treatments. It can be concluded that the different breeds of rabbit and inclusion of FCPHM in their diets significantly altered the composition and population of caecal microbiota.

Keywords—Caecum, Cocoa pod husk, Microbial flora, Rabbits.

I. INTRODUCTION

In Nigeria presently the demand for animal protein is far higher than the supply, thus for quick increased supply of animal protein and products, it is necessary that animals with short generation intervals be reared. One of the domestic animals with short generation interval considered in this study is the domestic rabbit (Oryctolagus cuniculus).

In terms of commercial production, the rabbit excels other livestock animals like cattle, sheep and goat and ranks close to broiler chicken in terms of growth rate, feed conversion efficiency and meat quantity (Adegbola et al., 1986). Nutritionally, rabbit meat is considered the healthiest meat option containing a higher protein (20-21%) and lower fat content (10-11%) when compared with meat from other species (Ajayi et al., 2007). Furthermore, Janieri (2003) had reported that rabbit meat has the cholesterol value of 169mg/100g (dry matter basis) when compared with beef (200mg), chicken (220mg), and low sodium content. Consequently, rabbit meat has been listed in United State
Department of Agriculture (USDA) as an approved meat source for hypertensive patients.

However, as reported by Ozor and Madukwe (2005) nutrition and housing are some of the constraining factors in the adoption of improved rabbit technologies by small-scale farmers, with similar observations being made by Oseni, et al. (2008) in western Nigeria. Therefore, the resurgence of interest in rabbit production in Nigeria calls for research into alternative sources of energy and protein yielding ingredients to replace or supplement the expensive conventional cereal grains and legumes. The prices of such conventional protein and energy feed ingredients such as maize, rice, sorghum, ground nut, soybean have escalated over time that it is becoming uneconomical to use them in rabbit feed (Esonu et al., 2004; Oduguwa et al., 2004). Animal nutritionists have therefore advocated for the use of agro-industrial by-products as unconventional feedstuffs because they are cheaper and available in large quantities in producing countries.

Several crops and their by-products have potential as possible alternatives for livestock feed industry. One such crop is cocoa, a very abundant crop in tropical regions of Africa and its by-products have been successfully used as alternative feedstuff in livestock production (Makinde et al., 2019). Cocoa pod husk, cocoa bean shell and cocoa bean meal form over 70% (w/w) of a whole matured fruit of cocoa (Theobroma cacao L.), and these are the major agro-industrial by-products from cocoa processing industries and are usually considered as “waste” and left to rot on the cocoa plantation.

Diet plays an important role in modulating gut microbiome by providing food substrates for gut microorganisms (Conlon and Bird, 2014; Kim et al., 2015). Furthermore, several studies demonstrated that a close relationship exists between gut microflora and health of host. The role of indigenous microorganisms includes both a protection against pathogens (the barrier effect) and a strong implication in the development and maturation of digestive mucosa immunity. Also the maintenance of gut health is complex and relies on a delicate balance between the mucosa (including the absorptive epithelium and the digestive immune system), the commensal microflora and environmental factors including diet (Fortun-Lamothe and Boullier, 2004).

Hence this study was carried out to determine the effect of inclusion of CPH meal in diets on caecal microbiome and inadvertently the health status of two breeds of rabbits.

II. MATERIALS AND METHOD

Experimental site
The feeding trial of the experiment was carried out at the Livestock section (Rabbit unit) of the Teaching and Research Farm and laboratory analysis in Microbiology Laboratory of the Department of Animal Production and Health both of The Federal University of Technology, Akure, Nigeria.

Collection and Fermentation of Cocoa Pod Husk

The cocoa pod husks (CPH) were collected from cocoa plantations in Ibadan and Ondo towns, Nigeria. The pods were cleaned with sterile water, chopped, sun-dried, milled and analyzed for proximate composition. The milled cocoa pod husk (CPH) was then subjected to solid state fermentation using Rhizopus stolonifer to reduce the theobromine and fibre contents prior to its usage. The fermentation process was carried out by dissolving ten (10) grams of urea in 10 litres of water which was used to moisten the CPH meal. One litre of the prepared inoculums of the starter culture of Rhizopus stolonifer was used to inoculate the urea treated CPH meal and kept in a tray incubating chamber to initiate the fermentation process. The fermentation of the cocoa pod husk meal was terminated on the 14th day followed by sun drying of the substrates for 5 - 7 days to inactivate the microorganisms. The dried CPH meal was subsequently kept in air-tight plastic container while a sample was taken for post-fermentation proximate analysis.

Experimental Animals and Arrangement

Sixty 5 weeks (35 days) old grower rabbits comprising thirty New Zealand White breed and thirty Chinchilla breed were purchased from a reputable farm in Ogun State, weighed individually and grouped into treatments after balancing for weights and penned individually in their hutches using completely randomized design for eight experimental weeks. There were ten replicates per treatment with one rabbit per replicate. Weekly weights and feed intake of each rabbit were measured.

Experimental Diets

Three experimental diets were formulated to meet the nutritional requirements of the grower rabbits in which fermented cocoa pod husk meal (FCPHM) was incorporated into the feed as the test ingredient at varied levels of 0.0, 12.5 and 25.0% which were designated as Diet I (control), Diet II and Diet III respectively. The animals were provided feed and water ad libitum throughout the eight-week experimental period. The gross composition of the experimental diets for the rabbits is presented in Table 1.
Table 1: Gross composition of the experimental diets for rabbits.

| Ingredients        | Diet I | Diet II | Diet III |
|--------------------|--------|---------|----------|
|                    | 0.0%   | 12.5%   | 25.0%    |
| Maize              | 18.40  | 21.10   | 19.40    |
| GNC                | 8.70   | 7.80    | 4.60     |
| PKC                | 25.50  | 10.80   | 7.40     |
| SBM                | 4.50   | 4.30    | 3.10     |
| Wheat offal        | 6.90   | 3.40    | 0.40     |
| GNH                | 34.20  | 38.50   | 38.50    |
| **FCPH**           | **0.00** | **12.50** | **25.00** |
| Vegetable Oil      | 0.80   | 0.80    | 1.10     |
| Lysine             | 0.10   | 0.10    | 0.10     |
| Methionine         | 0.10   | 0.10    | 0.10     |
| Limestone          | 0.40   | 0.30    | 0.10     |
| Premix             | 0.20   | 0.15    | 0.10     |
| Salt               | 0.20   | 0.15    | 0.10     |
| **Total**          | 100.00 | 100.00  | 100.00   |

**Calculated Composition**

|                     | Diet I | Diet II | Diet III |
|---------------------|--------|---------|----------|
| Dry Matter, DM (%)  | 90.15  | 89.87   | 89.29    |
| Metabolisable Energy, ME (Kcal/kg) | 2505.89 | 2506.44 | 2501.95 |
| Crude Protein, CP (%) | 15.92  | 15.95   | 15.68    |
| Crude Fiber, CF (%)  | 15.64  | 15.05   | 15.04    |
| Calcium, Ca (%)      | 0.94   | 0.93    | 0.82     |
| Av. Phosphorus, P (%)| 0.69   | 0.64    | 0.59     |
| Lysine (%)           | 0.87   | 0.88    | 0.79     |
| Methionine (%)       | 0.60   | 0.59    | 0.59     |

**Analysed Composition**

|                     | Diet I | Diet II | Diet III |
|---------------------|--------|---------|----------|
| Dry Matter, DM (%)  | 88.30  | 88.90   | 87.86    |
| Crude Protein, CP (%)| 16.38  | 16.47   | 16.01    |
| Crude Fiber, CF (%) | 15.62  | 15.07   | 14.95    |
| Ash (%)             | 4.51   | 7.33    | 7.18     |
| Ether Extract (%)   | 6.32   | 5.14    | 5.06     |
| Nitrogen-Free Extracts (%) | 45.47 | 44.89   | 44.66    |

SBM = Soybean meal, PKC = Palm kernel cake, GNC = Groundnut cake, GNH = Groundnut husk, FCPH = Fermented cocoa pod husk.
Sample Collection

Two rabbits were taken from each dietary treatment group and humanely slaughtered. The animals were dissected and the gastrointestinal tract was located, then was removed. Samples of the caecal content for microbial analysis were collected in a sterile manner from approximately 3 cm from the ileocecal junction. The following analysis were carried out on the samples collected.

Microbiological Analysis

Bacterial isolation and determination of total viable counts

A portion of each sample (1g) taken from the caecum of experimental rabbits was added into test tubes containing sterile distilled water (9ml) and was thoroughly mixed to serve as stock. Four fold serial dilutions (10^1 to 10^4) of the stock was done using 1ml stock homogenate and 9mls sterile distilled water in order to obtain discrete colonies (Moshood et al., 2012). The media (Nutrient Agar) used was prepared from commercially dehydrated products and reconstituted according to the manufacturer’s directives, sterilized and allowed to cool. 1ml each of the serially diluted sample was dropped at the centre of a Petri-dish followed by pouring of the nutrient agar using the pour plate method as described by Mumtaz et al. (1986). It was allowed to solidify for some minutes and then incubated at 37 °C for 24 hours. The colonies that emerged were counted and calculation for the colony forming units were expressed as log cfu/ml using the formula as described by Rukayya et al. (2016).

Identification and characterization of bacterial isolates

The bacterial colonies that developed on the nutrient agar plates were sub-cultured by streaking on freshly prepared nutrient agar plates and MacConkey agar plates until pure colonies were obtained according to the conventional procedure as highlighted by (Fawole and Oso, 2001). Then isolates were characterized and identified based on their morphological and cultural characteristics including shape, size, pigmentation, elevation and marginal characteristics of the colony and Gram staining. Then a series of biochemical reactions which include oxidase test, catalase test and coagulase test were done. Sugar fermentation assay and indomethyl red tests were also carried out as stated by Olutiola et al. (1999).

Statistical Analysis

All data collected were subjected to two – way analysis of variance (ANOVA) using Statistical Package for Social Sciences (SPSS, version 23) and where significant differences were found, the means were separated using Duncan Multiple Range Test of the same statistical package.

Table 2: Bacterial and Coliform counts isolated from caecum of New Zealand White and Chinchilla rabbits fed diets containing FCPHM

| Factors | Total Bacterial Count (×10^2 cfu/ml) | Total Coliform Count (×10^2 cfu/ml) |
|---------|-------------------------------------|-------------------------------------|
| Breed   | NZW                                 | 93.67^a                            | 13.58^a                            |
|         | CHL                                 | 70.58^b                            | 8.56^b                             |
|         | SEM                                 | 7.18                                | 1.15                                |
|         | P-value                             | 0.036                               | 0.006                               |
| FCPHM   | Control                             | 56.88^b                            | 7.38^b                             |
| Inclusion levels | 12.5%                             | 87.82^a                            | 10.63^b                            |
|         | 25%                                 | 101.63^a                           | 15.25^a                            |
|         | SEM                                 | 8.80                                | 1.41                                |
|         | P-value                             | 0.006                               | 0.003                               |
| Interaction Effect (Breed X FCPHM Inclusion Level) | NZW Control                      | 55.25                               | 9.25                                |
|         | 12.5%                               | 101.25                              | 15.50                               |
|         | 25%                                 | 124.50                              | 16.00                               |
|         | CHL Control                         | 58.50                               | 5.50                                |
III. RESULTS

Bacterial and Coliform Counts

Tables 2 shows the total viable bacterial and coliform counts obtained from samples in the caecum of the New Zealand White and Chinchilla rabbits fed the different experimental diets. It revealed that the breed effect had significant (P<0.05) influence on the bacteria and coliform (P<0.01) counts. The chinchilla rabbit had fewer bacterial (70.58 × 10^2 cfu/ml) and coliform (8.58 × 10^2 cfu/ml) counts than the New Zealand White rabbits. The dietary inclusions of FCPHM also had significant effect (P<0.05) on the bacterial and coliform counts and the least count was recorded in the control group (56.88 × 10^2 cfu/ml) followed by the group fed diet containing 12.5% FCPHM (87.88 × 102 cfu/ml) and then the 25% FCPHM group (101× 10^2 cfu/ml). The interaction effect between the breed and varying dietary treatment factors had no significant effect (P>0.05) on both bacterial and coliform counts.

Percentage distribution of bacteria organisms isolated from caecum of experimental rabbits

The varying diets significantly (P<0.05) affected the abundance and richness of certain types of bacteria detected in the experimental rabbits.

Detailed percentages of the bacteria organisms isolated from the caecum of the Chinchilla rabbits fed the different levels of dietary FCPHM is shown in Figures 1A-C. Only Salmonella spp. and Bacillus cereus were commonly isolated from the rabbits fed the three dietary treatments while Aerobacter cloacae, Enterobacter spp and Klebsiella spp were further isolated from the control group; Aerobacter cloacae, Enterobacter spp and Bacillus subtilis were isolated from the 12.5% FCPHM group while Staphylococcus faecalis, Proteus spp, Bacillus subtilis and Klebsiella spp were further isolated from the 25% FCPHM group.

Figures 2A-C shows that Klebsiella spp, Salmonella typhi and Bacillus cereus were present in the caecum of the New Zealand White rabbits in all the treatment groups. Staphylococcus epidermidis, Enterobacter spp and Bacillus subtilis were also found in rabbits fed the control diet and the 12.5% FCPHM supplemented diet while Proteus spp and Staphylococcus epidermidis were further isolated from those fed 25% FCPHM supplemented diet.
Fig. 1B: Percentage distribution of bacteria isolated from caecum of Chinchilla rabbits fed diet containing 12.5% FCPHM

Fig. 1C: Percentage distribution of bacteria isolated from caecum of Chinchilla rabbits fed diet containing 25% FCPHM

Fig. 2A: Percentage distribution of bacteria isolated from caecum of Newzealand Rabbits fed control diet
IV. DISCUSSION

The rabbit enteric microbiota plays a key role in maintaining rabbit health, including helping to digest forage-based diet and aiding in immune system regulation and development (Kylie, 2016).

This present study revealed the presence of a wide variety of bacteria in the caecum of the experimental rabbits. Earlier speculation has been made that rabbit caeca contain a large proportion of undescribed bacterial species (Michelland et al., 2010) and this suggested that the composition of these novel species may vary between individuals which was further buttressed by the report of North et al. (2019).

In this study for the overall bacterial communities, the bulk of the bacterial population across breeds and diets were observed to be Bacillus, Salmonella, Klebsiella, Enterobacter, Staphylococcus spp, Aerobacter and Proteus. However, a high percentage of reads across samples were assigned to Bacillus species and Salmonella organisms which suggests that the richness of certain bacteria was significantly affected by diet as opined by Zhu et al. (2015).

It was noted that the bacteria Lactobacillus genus was not isolated from the gut of experimental rabbits which is in line with report by Fortun-Lamothe and Boullier (2004) that the absence of the genus Lactobacillus in the rabbit flora is original. Also Zhu et al. (2015) reiterated the absence of the genus Lactobacillus in the rabbit flora as unique and being in accordance with previous data obtained by Yu and Tsen (1993) with culture-based methods. Penney et al. (1986) previously hypothesized that this is due to highly acidic environment in the GIT of adult rabbits.

Also, this present study revealed the absence of Escherichia coli in the gut of adult rabbits and this is similar to reports of
large-scale studies that the cultivable fraction of rabbit digestive microbiota in healthy adults is characterized by the absence or low density of *Escherichia coli* (Yu and Tsen 1993; Pupoe et al. 1997). Fortun-Lamothe and Boullier (2004) also made similar observations that *Escherichia coli* reached a maximum level at the 2nd or 3rd week of life and then decreased to be *residual* absent after weaning in the gut of rabbits.

It was further discovered in this present study that the microbial abundance varied with the different nutritional treatments and breeds of rabbits. This may be due to the fact that an important fraction of the diets enters the caecum, substrates for microbial fermentation and a change in diet composition can modify the nature of the digestate be fermented in the caecum and, consequently, can affect microbiota composition and activity as suggested by Jehl and Gidenne (1996). Previously, a study by Bogone-Vantuset et al. (2014) reported that microbes present in the gastrointestinal tract are a direct function of the nutrition of the rabbits. This suggests that the FCPHm inclusion in the diets of the experimental rabbits aided the proliferation of different types of bacteria organisms. This is further buttressed by the work of Michelland et al. (2011) which demonstrated that the bacterial communities of the rabbits caecum change and adapt rapidly to reach a new equilibrium in response to nutrition. However, in a particular study, the impact of dietary composition on gut microbiota was not reported (Massip et al. 2012).

Also, in line with this present study where a variety of bacterial organisms was found in the caecum of the experimental rabbits, Combes et al. (2013) asserted that an abundant bacterial community is present throughout the caecum-colon and in the hard and soft faeces (10^{10} – 10^{12} bacteria/bag) of rabbits.

The diverse and abundant bacterial population reported in the caecum of experimental rabbits probably as a result of the varying dietary treatments were also pronounced on the bacterial and coliform counts whereas the combined effects of breed and dietary treatments were not pronounced on the bacterial and coliform counts.

The bacteria and coliform counts in caecum of New Zealand White rabbits were found to be more than that of Chinchilla rabbits which can be attributed to breed effect, while the rabbits fed with varying levels of FCPHM were found to have higher counts of bacteria compared with those fed the control diet without FCPHM. The result also showed that the higher the level of inclusion of FCPHM the higher the bacteria and coliform counts recorded. This may be as a result of the high fibre content in the diet with the highest inclusion level of FCPHM meal which is in line with reports of previous studies that in animals fed a high-fibre diet, bacteria were found in the highest abundance compared with animals fed diets with lower fibre content (Gidenne and Bellier 2000; Gidenne and Fortun-Lamothe 2002). Similarly, Gidenne et al. (2004) suggested that the total bacterial biomass production was 3-fold higher for rabbits fed a high fibre/starch ratio. Though, Zhu *et al.* (2015) suggested that an unbalanced diet with excessive fibre or starch reduces microbial richness and diversity. Earlier studies had also reported that dietary starch/fibre was long thought to be a factor that predisposed rabbits to the development of undesirable microbiota (Cheeke and Patton. 1980).

The low coliform bacteria counts relative to the total bacteria counts ratio in the FCPH meal fed rabbits suggested that the caecum was predominantly colonized by non-pathogenic bacteria and toxic substances of pathogens were inhibited by gut beneficial bacteria as earlier hypothesized by Phuoc and Jamikorn (2017). It may also be due to the fact that acetic acid produced by resident bacteria during fermentation process is able to penetrate into bacterial cytoplasm resulting in a reduced internal bacterial pH and collapse of the electrochemical proton gradient which leads to bacteriostasis and death of susceptible bacteria such as caecal coliforms (Eklund, 1989).

Overall, this large bacterial community in the gut influences the overall health status of the rabbits as well as imparts on the digestion process in the animals. This is ascertained because short-chain fatty acids (acetate, butyrate, and propionate) which are metabolic end products from bacteria such as those present in the gut of the experimental rabbits are pivotal in several host physiological functions, such as nutrient acquisition, immunity, cell signalling, proliferation control, and pathogen protection as reported earlier by Tremaroli and Bäckhed (2012).
V. CONCLUSION

The inclusion of fermented cocoa pod husk meal (FCPHM) in feed of New Zealand and Chinchilla breeds of rabbit enhanced number of gut beneficial bacteria populations which could improve caecal fermentation and ultimately lead to better gut health and growth performance. In addition, caecal coliform population were reduced in the experimental rabbits. Also, the varying diets caused the proliferation of different types of bacteria in the gut.

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