Gut Micro-flora, Serum Bio-markers, and Growth Performance of Broiler Chickens Fed Supplemental Levels of *Bacillus* protease

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Abstract**—** To maintain a high quality and low fibre content of broiler feeds may require an external influence, such as the *Bacillus* protease to overcome some of the potential limitations imposed by vegetable protein diets. There is paucity of information on serum biomarkers, and gut micro-flora composition of chickens fed dietary *Bacillus* protease. The objective was to test the effect of dietary *Bacillus* protease inclusion on gut micro-flora, serum bio-markers, and growth traits of chicken. A total of 300 – day – old "Cobb 500" chicks were randomly assigned to five dietary treatments with five replicates of 12 birds each. The treatments include; PROT0 (0 g/kg; control), PROT10 (1 g/kg), PROT15 (1.5 g/kg), PROT20 (2 g/kg) and PROT25 (2.5 g/kg). The results showed that serum protein and albumin increased ($p<0.05$) while aspartate aminotransferase (AST) and alanine aminotransferase (ALT) decreased ($p<0.05$), as the amount of *Bacillus* protease increased. Bacteria population did not differ ($p>0.05$). Daily feed intake and the feed intake at starter phase, finisher phase, and overall phase decreased ($p<0.05$) as the levels of protease increased. Birds fed PROT25 had the highest ($p<0.05$) weight gain and a better feed conversion ratio (FCR) throughout the feeding trial. It was concluded that 2.5g/kg *Bacillus* protease can be safely included for improved weight gain, FCR, carcass yield, retail cut yields and overall health status of chickens.

Keywords: *Bacillus* protease, broiler chicks, carcass yield, micro-flora.

I. Introduction

Poultry production is a significant component of the economy and supply of animal protein, which remains grossly insufficient in developing countries. Iyayi and Davis [1] suggested that the supply of poultry products in poorer countries could be effectively and rapidly expanded to meet the growing need for animal protein. This is technically feasible because poultry are easily adaptable to most areas of the world, they have rapid gestation and development durations, with high muscle (flesh) tissue deposits. However, one of the biggest challenges to commercial poultry production is the availability of quality feeds at sustainable and stable prices. Kamel *et al*. [2] pointed out that protein is less digestible (80 - 85%) than starch (90%) in corn-soy diets, and that certain amounts of protein travel through the gastrointestinal tract without being completely digested [3]. Thus, the use of the mono-component protease enzyme may offer an opportunity to overcome some of the potential limitations imposed by vegetable – protein – based diets that may antagonize the serum biomarkers and growth of broiler birds [4]. Nutritional additives should be geared towards producing positive effects on the animal, promoting its health status and productive function while minimizing deleterious side – effects. Several serum enzymes are considered biomarkers that can be used to study liver and kidney functions. Elevated serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) concentrations indicate the release of aminotransferase from the cytoplasm to the blood stream, probably due to a damaged liver or differences in other tissues. Ahmad *et al*. [5] found that supplementations of protease had no adverse effect on the liver enzymes ALP, AST, and ALT and on total bilirubin concentrations. Xylanase and protease supplementation in broiler birds had no adverse effects on liver, kidney and various other internal organs [6]. Dietary multi-enzyme supplementation did not affect the serum alkaline phosphatase concentration of broiler birds [7]. The growth yield is closely linked to the nutrition quality of broilers, animals with an adequate supply and diversity of nutrients will promote the development of muscle tissues [8]. The addition of protease has been reported to improve the diet's protein digestibility and enhance nutrient availability for conversion of feed to meat yield [9], [10]. Exogenous protease supplementation can change the nutritional status and improve the growth of broiler chickens fed a corn-soybean meal diet [11]. Nutritional status is an important factor in the regulation of plasma hormones and intermediary metabolism in broiler birds [12], [13]. There is little information on serum biomarkers, and micro-flora composition of broiler birds fed dietary *Bacillus* protease. Therefore, this study sought to examine the effects of *Bacillus* protease supplementation on growth traits, gut micro-flora and serum biochemical profiles of broiler chickens fed maize-soybean meal diets.

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II. Materials and Methods

A. Ethical statement

Ethical principles were taken into consideration during the study to adhere to the national and international standards governing research of this nature with regards to the use of research animals.

B. Study site

This study was conducted at the poultry unit of North-West University experimental farm (Molelwane), in the North-West province of South Africa. The study duration was six weeks.

C. Enzyme characteristics

The tested protease enzyme (RONOZYME® ProAct (RPA), DSM Nutritional Products Johannesburg South Africa) is a granulated heat-stable formulated product from *Bacillus strain E A.1* with an enzyme activity of 75,000 PROT/g. One PROT is one protease unit, and is defined as the amount of enzyme that releases 1 mmol of p-nitroaniline from 1 mM substrate (Suc-Ala-Ala-Pro-Phe-pNA) per Adler minute at pH9.0 and 37°C. The *Bacillus* enzyme was selected as feed enzyme candidate because of its bioactive intrinsic characteristics. According to the manufacturer, at peptic and acidic conditions (pH), the enzyme retains more than 90% residual activity after 2hrs at 40°C (DSM).

D. Experimental diets

Iso-nitrogenous and iso-caloric experimental maize-soybean meal diets were used in this study. The feeding strategy consisted of starter (0 – 21 d) and finisher (22 – 42 d) basal diets (Tables 1 and 2), which were formulated to meet the birds' dietary nutrient requirements (NRC, 1994). At each feeding phase (i.e., starter and finisher) five dietary treatment were formulated by the addition of the *Bacillus* protease at five varying levels. The five experimental diets generated were Protease0, Protease10, Protease15, Protease20 and Protease25 represented as PROT0 (only basal diet; BD), PROT10 (BD + 1 g protease/kg diet), PROT15 (BD + 1.5 g protease/kg diet), PROT20 (BD + 2 g protease/kg diet) and PROT25 (BD + 2.5 g protease/kg diet) respectively for both starter and finisher diets. The ingredient and chemical composition of the five experimental diets for the starter and finisher phases are presented in Tables 1 and 2, respectively. The chemical (proximate) composition of the experimental diets was analyzed according to AOAC (2006) methods with average crude protein and metabolizable energy of 23.70 CP and 12.60 MJ of ME/kg respectively for starter chicks while an average of 19.70 CP and 13.00 MJ of ME/kg was recorded for finisher birds.

TABLE I. INGREDIENT (%) AND CHEMICAL COMPOSITION (G/KG DM UNLESS OTHERWISE STATED) OF EXPERIMENTAL DIETS FOR BROILER CHICKS AT THE STARTER PHASE (0 – 3 WEEKS)

Ingredient	**PROT0**	**PROT10**	**PROT15**	**PROT20**	**PROT25**
Yellow maize	62.98	62.98	62.98	62.98	62.98
Soybean meal	27.38	27.38	27.38	27.38	27.38
Sunflower meal	4.00	4.00	4.00	4.00	4.00
Fish meal	2.50	2.50	2.50	2.50	2.50
Canola oil	0.11	0.11	0.11	0.11	0.11
Limestone	1.32	1.32	1.32	1.32	1.32
MonoCaP	0.44	0.44	0.44	0.44	0.44
Salt	0.28	0.28	0.28	0.28	0.28
Methionine	0.23	0.23	0.23	0.23	0.23
Threonine	0.05	0.05	0.05	0.05	0.05
Lysine	0.32	0.32	0.32	0.32	0.32
Choline Cl	0.10	0.10	0.10	0.10	0.10
[1]VMP	0.20	0.20	0.20	0.20	0.20
[2]Maxiban	0.05	0.05	0.05	0.05	0.05
[3]Surmax	0.04	0.04	0.04	0.04	0.04
Protease	0.00	0.10	0.15	0.20	0.25
Total	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**
Chemical composition					
Moisture	11.11	10.89	11.00	11.08	11.01
ME (kcal/kg)	2988.96	2984.86	2987.06	2982.99	2985.69
Crude protein	23.83	23.90	23.79	23.85	23.88
Crude fat	4.01	4.03	3.99	4.11	4.08
NDF	15.32	15.27	15.39	15.28	15.30
ADF	3.85	3.95	3.97	4.00	4.02
Calcium	0.93	0.94	0.92	0.93	0.94
Phosphorus	0.66	0.68	0.65	0.69	0.67

MonoCaP = Monocalcium Phosphate. Choline Cl = Choline Cloride. VMP = Vitamine mineral premix. ME = Metabolizable energy. NDF = Neutral detergent fibre. ADF = Acid detergent fibre. PROT0 (only basal diet; BD), PROT10 (BD + 1g protease), PROT15 (BD + 1.5g protease), PROT20 (BD + 2g protease) and PROT25 (BD + 2.5g protease). [1a]2.5 kg of vitamin premix contained: 2700 mg retinal, 400 mg calcidiol, 18 g tocopheryl acetate, 2000 mg menadione, 1800 mg thiamine, 6600 mg riboflavin, 10 g niacin, 30 g calcium pantothenate, 3 g pyridoxine, 1 g folic acid, 15 mg cobalamin, 250 g choline chloride, 100 mg biotin. [b]2.5 kg of trace mineral premix contained: 100 g Mn, 50 g Fe, 100 g Zn, 10 g Cu, 1 g I, 200 mg Se. [2]1000g of Maxiban contained: 80 g/kg narasin, 80 g/kg nicarbazin. [3]1000g of Surmax contained: 100g/kg avilamycin

E. Experimental birds and management

A total of 300 one-day-old mixed-sexed broiler birds (Cobb 500®) were used in this study. Sixty birds (five replication of 12 birds in each replicate per treatment group) were assigned randomly to one of the five experimental diets (PROT0, PROT10, PROT15, PROT20 or PROT25). Each experimental diet was replicated in five experimental pens with 12 birds per pen measuring (2.5 m length × 2.5 m width × 2.5 m height). The birds were housed in cages with wood shavings as litter and they were provided with feed and water *ad libitum* throughout the six-week feeding period.

TABLE II. INGREDIENT (%) AND CHEMICAL COMPOSITION (G/KG DM UNLESS OTHERWISE STATED) OF EXPERIMENTAL DIETS FOR BROILERS AT THE FINISHER PHASE (4 – 6 WEEKS).

Ingredient	**PROT0**	**PROT10**	**PROT15**	**PROT20**	**PROT25**
Yellow maize	72.36	72.36	72.36	72.36	72.36
Soybean meal	24.55	24.55	24.55	24.55	24.55
Canola oil	0.24	0.24	0.24	0.24	0.24
Limestone	1.25	1.25	1.25	1.25	1.25
MonoCaP	0.17	0.17	0.17	0.17	0.17
Salt	0.39	0.39	0.39	0.39	0.39
Methionine	0.21	0.21	0.21	0.21	0.21
Tryptophan	0.05	0.05	0.05	0.05	0.05
Threonine	0.05	0.05	0.05	0.05	0.05
Lysine	0.34	0.34	0.34	0.34	0.34
Choline Cl	0.10	0.10	0.10	0.10	0.10
[1]VMP	0.20	0.20	0.20	0.20	0.20
[2]Maxiban	0.05	0.05	0.05	0.05	0.05
[3]Surmax	0.04	0.04	0.04	0.04	0.04
Protease	0.00	0.10	0.15	0.20	0.25
Total	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**
Chemical composition					
Moisture	11.01	11.03	10.97	11.10	11.13
ME (kcal/kg)	3110.94	3115.76	3118.00	3111.85	3113.93
Crude protein	19.75	19.91	19.70	19.83	19.85
Crude fat	4.08	4.12	4.12	4.15	4.09
NDF	15.93	18.32	18.28	18.21	18.02
ADF	4.85	4.97	5.02	4.95	5.00
Calcium	0.94	0.92	0.94	0.93	0.93
Phosphorus	0.61	0.62	0.61	0.61	0.61

MonoCaP = Monocalcium Phosphate. Choline Cl = Choline Cloride. VMP = Vitamine mineral premix. ME = Metabolizable energy. NDF = Neutral detergent fibre. ADF = Acid detergent fibre. PROT0 (only basal diet; BD), PROT10 (BD + 1g protease), PROT15 (BD + 1.5g protease), PROT20 (BD + 2g protease) and PROT25 (BD + 2.5g protease). [1a]2.5 kg of vitamin premix contained: 2700 mg retinal, 400 mg calcidiol, 18 g tocopheryl acetate, 2000 mg menadione, 1800 mg thiamine, 6600 mg riboflavin, 10 g niacin, 30 g calcium pantothenate, 3 g pyridoxine, 1 g folic acid, 15 mg cobalamin, 250 g choline chloride, 100 mg biotin. [b]2.5 kg of trace mineral premix contained: 100 g Mn, 50 g Fe, 100 g Zn, 10 g Cu, 1 g I, 200 mg Se. [2]1000g of Maxiban contained: 80 g/kg narasin, 80 g/kg nicarbazin. [3]1000g of Surmax contained: 100g/kg avilamycin

F. Serum biochemical profile

At 42 days of age, five birds were randomly selected from each experimental pen, and 2 ml of blood was collected from the wing vein using a sterile syringe and needles. The blood collected was transferred into a labelled treated vacutainer tubes. Red-top tubes without anticoagulant were used for serum biochemical analysis.

G. Growth performance

Average daily feed intake (ADFI) per bird was measured from day 1 to day 42 of age by subtracting the weight of the feed remaining from that of the feed initially supplied, and dividing the difference by the total number of birds in the pen. Average live-weight was measured weekly by weighing all the birds in each pen using a 10,100 g (10.1 kg) capacity precision weighing balance with model, A and D Weighing GF-10K industrial balance, made in Japan. The feed conversion ratio (FCR) was calculated as follows: = Feed intake / weight gain**,** it is the mathematical relationship between the input of the feed that has been fed to the bird and the weight gain of the bird**.** FCR can provide a good indication of how efficient a feed or a feeding strategy can be.

H. Cecal and ileum micro-flora composition

Five birds per treatment at the age of 42 days were killed by severing the jugular vein. The abdominal cavity was opened, and the entire gastro intestinal tract was removed aseptically. All digesta contents of ileum, caecum and colon were collected immediately under aseptic conditions into sterile glass bags and put on ice before they were transported to the laboratory for enumeration of microbial populations.

I. Statistical design and analysis

Data collected during the study were subjected to analysis of variance (ANOVA) for Completely Randomized Design (CRD) [14] using General Linear Model Procedure [15]. The statistical model used to test the effects of treatment on meat quality traits, carcass characteristics, serum biochemical profiles, and gut micro-flora is presented as follows: $Yij = \mu + Pi + Eij$. Where: Yij = Observed value of a dependent variable; μ = Overall mean; Pi = Effect of different levels of dietary *Bacillus* protease enzyme; and Eij = Residual error. The differences between means were tested for significance ($p<0.05$) using the LSD range test.

III. RESULTS

A. Serum biochemical profile

Serum biochemical parameters of broiler chickens fed maize-soybean meal diets with supplementary *Bacillus* protease are presented in Table 3. There were significant ($p<0.05$) differences between dietary treatments in terms of total protein, albumin, AST, and ALT, whereas urea, cholesterol, and alkaline phosphatase were not significantly ($p>0.05$) affected by diet. Total protein and albumin values increased ($p<0.05$) with increased levels of *Bacillus* protease supplementation. AST and ALT values decreased ($p<0.05$) as levels of *Bacillus* protease increased. Birds fed PROT0 or PROT10 had the highest ($p<0.05$) values of AST. Conversely, birds fed PROT15, PROT20, or PROT25 had the lowest ($p<0.05$) AST values. The highest values of ALT was seen in birds fed PROT0, while birds in other treatments (PROT10, PROT15, PROT20, and PROT25) had significantly lower ($p<0.05$) ALT values.

TABLE III. THE EFFECT OF DIETARY PROTEASE SUPPLEMENTATION ON SERUM BIOCHEMICAL PARAMETERS OF BROILER BIRDS

Treatment	**PROT0**	**PROT10**	**PROT15**	**PROT20**	**PROT25**	**SEM**	**P-value**
Parameters							
Protein (g/L)	27.50^{b}	29.20^{b}	33.20^{a}	32.80^{a}	30.80^{a}	0.43	0.04
Albumin (g/L)	11.50^{b}	11.50^{b}	13.10^{a}	12.90^{ab}	12.30^{ab}	0.12	0.02
Urea (mmol/L)	0.85	0.82	0.86	0.87	0.79	0.00	0.09
Cholesterol (mmol/L)	2.84	2.88	3.10	3.11	2.89	0.01	0.10
ALP (IU/L)	130.54	143.75	136.88	147.77	145.24	2.98	0.22
AST (IU/L)	331.18^{a}	320.30^{a}	278.22^{b}	269.30^{b}	265.85^{b}	6.87	0.03
ALT (IU/L)	15.33^{a}	12.63^{b}	12.57^{b}	12.99^{b}	11.59^{b}	0.13	0.01

[a,b,c,d]; Row means with different superscripts differ significantly. SEM= Standard error of the mean. AST = Aspartate aminotransferase. ALT = alanine aminotransferase. ALP = alkaline phosphatase. PROT0 = Basal Diet: BD (without PROT; protease). PROT10 = BD + 1 g PROT. PROT15 = BD + 1.5 g PROT. PROT20 = BD + 2 g PROT. PROT25 = BD + 2.5 g PROT.

B. Gut micro-flora composition

Table 4 shows the changes in caecum and ileum microbial population in birds fed corn-soybean meal diets with supplementary *Bacillus* protease. Although, different *Bacillus* protease levels used in the present study did not significantly ($p>0.05$) influence the bacteria population in the ileum and caecum, however, there was a progressive increase in the numerical values reported for *Lactobacillus* and *Bifidobacteria* in both ileum and caecum as the levels of *Bacillus* protease increased. On the other hand, even though the *E. coli* counts was not influenced ($p>0.05$) by different levels of *Bacillus* protease, there was a progressive drop/decrease in the numerical values reported for *E. coli* as the inclusion levels of *Bacillus* protease increased.

TABLE IV. THE EFFECT OF DIETARY PROTEASE SUPPLEMENTATION ON BACTERIA COUNTS (log_{10} cfu/g) IN THE ILEUM AND CAECUM OF BROILERS FED CORN-SOYBEAN MEAL DIETS.

Treatment	PRO T0	PRO T10	PRO T15	PRO T20	PRO T25	SEM	P-value
Ileum							
Lactobacillus	7.51	7.69	7.94	8.04	8.07	0.08	0.21
Bifidobacteria	5.19	5.30	5.49	5.64	5.67	0.03	0.17
E. coli	5.32	5.11	4.86	4.56	4.16	0.03	0.11
Caecum							
Lactobacillus	7.73	8.04	8.50	8.17	8.34	0.09	0.10
Bifidobacteria	6.97	7.07	7.11	7.53	7.59	0.06	0.09
E. coli	6.17	5.95	6.51	5.67	5.59	0.05	0.12

[a,b,c]; Row means with different superscripts differ significantly. SEM = Standard error of the mean. *E. coli = Escharichia coli.* PROT0 = Basal Diet: BD (without PROT; protease). PROT10 = BD + 1 g PROT. PROT15 = BD + 1.5 g PROT. PROT20 = BD + 2 g PROT. PROT25 = BD + 2.5 g PROT.

C. Growth performance

The effects of dietary *Bacillus* protease supplementation on the growth performance of broiler chicks are shown in Table 5. The day – old weight of broilers did not differ significantly ($p>0.05$) among the groups (i.e., treatments). Daily feed intake was highest ($p<0.05$) in birds fed PROT0 (101.67 g) and lowest ($P<0.05$) in birds fed PROT25 (82.72 g). Birds fed the highest amount of *Bacillus* protease (PROT25) recorded an improved ($p<.0.05$) daily weight gain of 72.62 g, while a daily weight gain of 64.35 g, 61.90 g, 60.87 g and 61.17 g were found for birds fed PROT0, PROT10, PROT15 and PROT20, respectively. During the starter phase, a lower ($p<.0.05$) feed conversion ratio (FCR) value was observed in birds fed PROT25 relative to the other treatments (PROT0, PROT10, PROT15 or PROT20). Body weight gain was highest ($p<.0.05$) for PROT25 fed birds yet birds fed PROT0, PROT10 or PROT20 consumed more feed ($p<.0.05$) than birds that received PROT25. At the finisher phase, the body weight gain of birds fed PROT25 was significantly higher ($p<.0.05$) than the gain of birds in the other treatment groups and similar with birds fed the control diet (PROT0). Birds fed PROT25 had the lowest ($p<.0.05$) FCR of 1.24 and an increased FCR of 1.75, 1.77, 1.76 and 1.76 was found in birds fed PROT0, PROT10, PROT15 or PROT20, respectively. Feed intake of birds fed the control diet (PROT0) was highest ($p<.0.05$), though similar to those that received PROT10 and PROT20, while PROT25 fed birds consumed less feed. The result showed that, the feed intake and FCR performance for birds during finisher phase maintained the same pattern of performance during the overall performance. As indicated in overall performance, birds fed PROT25 had a better ($p<.0.05$) body weight gain of 3050 g than those that received other treatments (2702.80g, 2599.80g, 2556.60g and 2569.20g for PROT0, PROT10, PROT15 and PROT20, respectively).

TABLE V. THE EFFECT OF DIETARY PROTEASE SUPPLEMENTATION ON OVERALL FEED INTAKE, BODY WEIGHT GAIN AND FEED CONVERSION RATIO OF BROILER BIRDS

Treatment	PROT0	PROT10	PROT15	PROT20	PROT25	SEM	P-value
Daily performance							
Day old weight (g)	44.00	45.00	45.00	44.00	45.00	0.12	0.22
Daily feed intake (g)	101.67^{a}	95.94^{ab}	93.36^{b}	95.55^{ab}	82.72^{c}	0.65	0.02
Daily weight gain (g)	64.35^{b}	61.90^{b}	60.87^{b}	61.17^{b}	72.62^{a}	0.15	0.04
Starter phase							
Feed intake (g)	1266.00^{a}	1211.00^{a}	1199.50^{ab}	1255.00^{a}	1087.80^{b}	1.75	0.04
Body weight gain (g)	943.40^{b}	941.20^{b}	940.80^{b}	956.20^{b}	1065.00^{a}	1.69	0.02
FCR (g/g)	1.34^{a}	1.29^{a}	1.27^{a}	1.32^{a}	1.03^{b}	0.01	0.03
Finisher phase							
Feed intake (g)	3004.00^{a}	2817.80^{ab}	2721.30^{b}	2758.90^{ab}	2386.50^{c}	3.75	0.02
Body weight gain (g)	1715.40^{ab}	1613.60^{b}	1570.80^{b}	1569.00^{b}	1940.00^{a}	1.98	0.04
FCR (g/g)	1.75^{a}	1.77^{a}	1.76^{a}	1.76^{a}	1.24^{b}	0.01	0.02
Overall performance							
Feed intake (g)	4270.10^{a}	4029.40^{ab}	3920.80^{b}	4013.10^{ab}	3474.40^{c}	4.46	0.02
Body weight gain (g)	2702.80^{b}	2599.80^{b}	2556.60^{b}	2569.20^{b}	3050.00^{a}	3.33	0.04
FCR (g/g)	1.79^{a}	1.59^{a}	1.57^{a}	1.59^{a}	1.16^{b}	0.01	0.01

[a,b,c]; Row means with common superscripts do not differ significantly. SEM= Standard error of the mean. FCR= Feed conversion ratio. PROT0 (only basal diet; BD), PROT10 (BD + 1g protease), PROT15 (BD + 1.5g protease), PROT20 (BD + 2g protease) and PROT25 (BD + 2.5g protease)

IV. DISCUSSION

A. Serum biochemical profile

Proteases play an important role in blood bio-markers as they it influence blood clotting and improve the immune system [16], [17]. Serum total protein and albumin increased along with increased levels of *Bacillus* protease addition. Protein in the serum comprises albumin and globulin, these proteins serve many diverse functions, including transport of lipids, hormones, vitamins and minerals for proper functioning of the immune system [18], [19]. The results of the present study corroborate those of Abudabo [20] and Allouche *et al*. [21], which suggest that the addition of enzymes increases protein digestibility. According to Cowieson and Ravindran [22], the energy and amino acid profile of maize-based diets for broilers can be enhanced by the addition of protease, amylase, and xylanase. Nevertheless, elevations in serum albumin and total serum protein were found when the level of dietary supplementation with *Bacillus* protease increased, which may reflect the ability of this enzyme to make available sufficient protein from the ingested diets compared to birds that consumed control diet. Previous studies have showed an increase in the protein digestibility in birds fed a corn-soybean meal diet supplemented with Avizyme® 1500 (protease and xylanase) [20]. Birds fed PROT0 had an increased ALT and AST values, but birds fed

PROT10 also recorded higher AST compared with birds fed higher levels of *Bacillus* protease (PROT15, PROT20 and PROT25). The presence of elevated ALT and AST levels are indicative of liver damage in broiler chicks, and thus comprises a valuable tool for determining a safe inclusion rate for feed additives, giving that diets may influence serum enzymes [23]. Some authors reported that elevated serum AST and ALT concentrations indicate the release of aminotransferase from cytoplasm to blood stream probably due to damage liver or different other tissues [5], [24]. This implies that birds fed diets without enzyme inclusion had adverse effect on hepatic cell and broiler health since higher concentration of AST and ALT indicates poor condition of some organs such as liver and kidney [18]. Enzyme supplementation significantly reduced the concentration of AST and ALT in chickens when compared to the control diet that had no enzyme. This findings is supported by those obtained by Lee *et al*. [25] and Mohamed *et al.* [23], who concluded that AST and ALT activities are indicative of liver and kidney damage in broiler chickens.

B. Gut micro-flora composition

In poultry, the micro-flora that colonizes the gastro-intestinal tract during the early post-hatch period forms a synergistic relationship with their host. Munyaka *et al*. [26] reported that the gut micro-flora performs an essential role in the nutrition and health of the host by encouraging digestion and absorption of nutrients, preventing pathogen's colonization, and shaping and keeping normal mucosal immunity. The major parameters for defining microbial structure and diversity are the richness and evenness of the bacteria. However, it is generally expected that dietary manipulations would influence the intestinal microbial structure and diversity [27]. According to Hubener *et al*. [28] and Masey *et al*. [29], the microbial population can be influenced by feed enzymes due to the changes imposed on the lumen contents. The non-significant differences recorded for bacteria populations in ileum and caecum are consistent with those of Gao *et al.* [13] who reported no significant change of *Lactobacillus* and Coliform bacteria counts in caecum content of 21-day-old birds. Luo *et al*. [6] did not find any effect of exogenous enzyme on counts of *Escherichia coli*, *Lactobacillus* and total aerobes in ileum and caecum of birds at 42d of age. Similarly, Yang *et al*. [30] reported no significant differences in the small intestine micro-flora counts in broilers fed a range of feed additives. Although, different *Bacillus* enzyme levels used in the present study did not significantly influence the bacteria population in the ileum and caecum, but there was a progressive increase in the numerical values reported for *Lactobacillus* and *Bifidobacteria* in both ileum and caecum as the levels of *Bacillus* protease increased. On the other hand, even though the *E. coli* counts was not influenced by different levels of *Bacillus* protease, there was a progressive drop/decrease in the numerical values reported for *E. coli* as the levels of *Bacillus* protease increased. These observations may be due to the enzyme's ability to increase the levels of available substrate for microbial fermentation which improve protein digestibility and the production of short chain fatty acids (SCFA) in the gut as a result of non-digested protein diet that become available to the gut micro-biota [31]. Nabizadeh *et al.* [32] also reported a similar observation. It is possible that *Bacillus* protease supplementation resulted in the numerical multiplication of beneficial bacteria in the present study. According to Jozefiak *et al*. [33], exogenous enzyme acts not only by lowering intestinal viscosity, but it also leads to the development of more competitive bacterial communities with higher intra-bacterial competition, which limits bacterial interference with nutrient absorption, and may contribute to the potential reduction of pathogenic population. Dietary factors can also influence microbial populations in birds. Some authors [34] observed higher population of *Escherichia coli* and *lactobacilli* in the digesta of broilers that consumed wheat and barley, relative to those that received corn diets. Changes in the intestinal micro-biota of chickens can alter the mucosal structure and thereby influence nutrient absorptive capacity [26]. Many researchers reported that increasing SCFA density causes a gradual decrease in the proliferation rate of *Enterobacteria* but not that of *Lactobacillus*, *Bifidobacteria* [35], [36], [32]. Contrary to the results of this study, exogenous enzymes have been reported to modulate the gut micro-biota of birds which may in turn affect the health status and the extent of digestion by the host [37].

C. Growth performance

In the present study, the supplementation of *Bacillus* protease had an effect on body weight gain (BWG), feed intake (FI), and FCR of chicken at all phases of growth. Birds that received dietary treatment PROT25 had higher BWG and improved FCR with less FI. The optimum results may be obtained with 2.5 g of this enzyme (i.e., the highest level of *Bacillus* protease used the present study); the other treatments may have adversely affected variations in feeding and BWG patterns. This is similar to the broiler performance reported by Ghazi *et al.* [38] who attributed the performance to improved true metabolizable energy and true nitrogen digestibility. Dietary fiber was suggested to be responsible for accelerating digesta passage rate [39], [40]. However, due to the low fiber content of dietary PROT25 as a result of the maximum enzyme activities, broilers that received PROT25 may have experienced lower digesta passage rate than those fed other dietary treatments. Previous studies [41] reported that the longer the low fibre digesta in the intestinal tract, the greater the chances for better feed digestion which thereby improves growth performance. According to Engberg *et al*. [42] and Selle *et al*. [43], whole wheat (high fibre) feeding

reduces the activities of amylase in the pancreatic tissue while the addition of protease increased chymotrypsin and lipase activities. The present results are also in line with the findings of Odetallah *et al*. [44] who reported that protease addition in a normal protein diet at 21 days of age resulted in a significant increase in BWG and decrease in feed intake. Similar results were reported by Odetallah *et al*. [45], who observed a significant increase in BWG and FCR in broilers supplemented with protease (versazyme) in high and normal protein diets. Kocher *et al*. [46] and Cowieson and Adeola [47] suggested that, improved BWG and FCR of birds fed dietary protease supplement may be attributed to the release of nutrients, making them available for utilization especially when feed ingredients are of low/inferior quality and/or with low bioavailability. Freitas *et al*. [48] noted a general decrease in FI of birds fed protease supplemented diets similar to the results obtained in the present study. The fact that chickens that received nutrient-dense diet fulfil their nutrient requirements by taking less amount of feed may explain why there was a decreased feed intake recorded for birds fed PROT25. This confirms the influence of protease in nutrients availability for chickens. Many authors share the same view [49], [50], [51]. Indeed, Hajati *et al*. [52] and Hajati [53] opined that enzyme supplementation might improve broiler performance by improving nutrient digestibility. This mechanism might be induced, at least partially, by a reduction of the viscosity due to decreased retention time of digesta in the gut [54]. Fru-Nji *et al.* [55] reported that protease supplementation, when included up to about 3% in broiler finisher, resulted in a non-significant improvement of BWG. Such incongruities may be due to differences in feed ingredient contents, levels of exogenous enzymes inclusion or breeds of chicken used [56].

V. CONCLUSION

We conclude that broiler chickens respond positively to supplementation with *Bacillus* protease with respect to gut micro-flora. *Bacillus* protease inclusion improved serum bio-markers, reducing AST and ALT activities that are indicative of liver and kidney damage in broiler chickens. In other words, the *Bacillus* protease protects the liver and kidney, which thereby stabilized the health status of the chickens. Summarily, birds fed 2.5 g/kg feed (PROT25) produced a better FCR and higher weight gain.

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Author Biography

Chika E. Oyeagu hails from the Eastern part of Nigeria, precisely, Enugu State. After attending primary and secondary education, he gained admission in Animal Science Department, University of Nigeria Nsukka, Nigeria in 2003. He graduated with B. Agric (Animal Science) in the year 2008. He enrolled for his Master's degree in the University of Nigeria Nsukka, Nigeria, in 2012, and graduated in 2014 with his specialty in Animal Biochemistry and Nutrition. He later gained admission for his PhD in the Department of Livestock and Pasture Sciences, University of Fort Hare, Alice, South Africa, in 2015, and graduated in 2019.

Chika worked at Folad Farms LTD in Edo State Nigeria as farm manager in 2009 – 2011. During his PhD studies (2016 - 2019), he served as a research assistance and he taught different Undergraduate and Honours modules. He is currently a Post-Doctoral Research Fellow in the Department of Agriculture, Cape Peninsula University of Technology, Wellington Campus, Cape Town South Africa. In the year 2019, Chika published 6 (six) papers in different DHET accredited journals. In 2020, three paper has been accepted in different DHET accredited journals, while three others has been accepted in DHET accredited conference proceedings. However, a number of manuscripts are still under review in different journals. Chika has a strong background in Agriculture, meat science, livestock nutrition, health and production.

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