Evaluation of optimum inclusion levels of *Bacillus subtilis* as replacement for antibiotic growth promoters in broiler chickens production under field conditions in Nigeria

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Abstract

A feeding trial was conducted to evaluate the response of broiler chickens fed diets supplemented with *Bacillus subtilis* as replacement for antibiotic growth promoters (AGPs). A total of 510 day old Ross broiler chicks were allotted randomly to five dietary treatments each replicated thrice, with 34 chicks per replicate. *Bacillus subtilis* was included at 0, 75000, 150,000 and 225,000cfu/100 Kg diet for T1-T4, respectively while T5 had Oxytetracycline. Parameters collected were on growth performance, Haematology, liver function, immune response, Antioxidant capacity, villi Morphometrics and ceacum microbial contents. All data were subjected to analysis of variance and significant differences among treatment means were compared using the Turkey test of significance. The results for the starter phase showed birds on the antimicrobials had significantly ($P < 0.05$) high final weight, weight gain, feed intake and better feed conversion ratio than the control. Chicks fed diet supplemented with 225,000 cfu *Bacillus subtilis* showed high performance in terms of final weight, weight gained, best and least feed cost per kg gain (N 235.2) and feed conversion ratio (1.60). Haematological indices were not significantly ($P > 0.05$) different for the starter phase while significant ($P < 0.05$) differences were observed in MCH (Mean corpuscular haemoglobin) and MCHC (Mean corpuscular haemoglobin Concentration). Liver function indices showed decreased concentration for Aspartate-amino transferase (AST) (33.30-43.30 μL) and Alkaline phosphatase (ALP) (72.30-85.80 μL respectively) for the *Bacillus subtilis* diets compared to control treatment values of 56.00 μL and 85.80 μL for AST and ALP respectively. The result for Immune response showed that the level of albumin to globulin ratio increased for birds fed levels of *Bacillus subtilis* and AGP than in the control after each administration of Gumboro and Lasota vaccines. Results for Antioxidant capacity showed that there were no Significant ($P > 0.05$) difference in all parameters measured except for Glutathione peroxidase (GPx). The GPx activity was significantly ($P < 0.05$) higher for birds fed levels of *Bacillus subtilis* when compared with the control and AGP. The result for the finisher phase showed that Birds fed diet supplemented with 75,000 cfu showed best performance in terms of final weight, feed consumed, feed conversion and had the least cost of production. Villi Morphometric showed significant ($P < 0.05$) differences in all parameters measured except for villi width. Villi area, height, width, crypt depth and villi height/crypt depth ratio were higher for birds fed levels of *Bacillus subtilis* and AGP than the control. Levels of *Bacillus subtilis* in the diet increased beneficial bacteria population and also prevent the colonization of the gut by pathogenic bacteria. It is concluded that *Bacillus subtilis* significantly improved growth of broiler chickens, feed conversion, significantly lowered cost of production even more than the AGPs; promotes antioxidant capability of birds; enhanced disease resistance by improving the immune response of broiler chickens. *Bacillus subtilis* is therefore a potential replacement for antibiotic growth promoters.

Keywords: *Bacillus subtilis*, Antibiotic growth promoter, Performance, broiler chickens

Introduction

Poultry serves as one of the means of satisfying the increased demand for animal protein. Presently, chicken meat is on demand as a source of protein with low cholesterol value, due to its generally low fat contents. Therefore, adaptation of broiler farming is increasing day by day by farmers. Feed as a major input item in broiler production for being 75% of the production cost has a vital role in broiler economics. Hence, it is imperative to give due attention to proper utilization of feed without adversely affecting the growth or production performance of broilers (Kokje, 1999).

The use of antibiotics as growth promoter intended not only as therapeutic but also as feed additive of continuous use in animal started appropriately five decades ago. Antibiotics have been also used to promote growth rate, improve feed conversion ratio (FCR) and reduce mortality in broiler flocks (Broz and Paulus, 2015). Usually, Antibiotic Growth Promoters (AGPs) are administered at low doses, absorbed minimally from the gut and when incorporated into the feed, they act by specifically reducing the number of pathogenic bacteria. However, repeated use of antibiotics in poultry diets resulted in severe problems like resistance of pathogen to antibiotics, accumulation of antibiotics residue in their products and environment, imbalance of normal microflora and

reduction in beneficial intestinal microflora (Barton, 2000). Due to these problems, in January 2006, the EU placed a total ban on the use of feed antibiotics. This total ban on the use of antibiotics as growth promoters has been integrated into a new EU regulation concerning feed additives (Broz and Paulus, 2015).

The Increase in bacterial resistance to antibiotics in both humans and livestock has caused an increase in public and governmental interest in eliminating sub-therapeutic use of antibiotics in livestock. This raises Concerns for food safety, environmental conservation and producing safer human foods from animal sources more efficiently and at lower cost. This has given impetus to continued search for new feed additives that would positively modulate the gut micro-flora, increase rate of growth and level of production (Dhama *et al.*, 2011; Youssef *et al.*, 2013). Probiotics are one of such alternatives to AGPS that have been evaluated with promising results. This study was therefore aimed at evaluating the optimum level of inclusion of a probiotic-Enviva[®]PRO 202 GT *Bacillus subtilis* strain in the diet of broiler chickens on growth performance, haematology, liver functions, immune response, antioxidant capacity, villi morphometrics and intestinal microbiota.

Materials and methods

Table 1: Effect of *Bacillus Subtilis* on the performance of broiler chicks 0-28 days

Parameters	0	75,000 cfu	150,000cfu	225,000cfu	Oxytetracycline	SEM
Initial weight (g/bird)	45.10	45.10	45.10	45.20	45.00	0.64
Final weight (g/bird)	1004.90 ^b	1065.50 ^a	1063.20 ^a	1074.70 ^a	1079.00 ^a	27.28
Weight gain (g/bird)	959.80 ^b	1020.30 ^a	1018.10 ^a	1029.50 ^a	10340 ^a	27.08
Feed intake (g/bird)	1681.80 ^b	1698.50 ^b	1731.10 ^a	1659.70 ^b	1761.10 ^a	25.16
Feed conversion ratio	1.80 ^b	1.70 ^b	1.70 ^b	1.60 ^a	1.70 ^b	0.03
Feed cost (₹/Kg)	145.20	145.40	145.70	145.70	152.50	0.00
Feed cost/gain (₹/Kg gain)	254.80 ^{ab}	242.30 ^{ab}	247.80 ^{ab}	235.20 ^a	260.00 ^{bc}	4.43
Mortality (%)	1.96 ^b	1.96 ^b	1.96 ^b	0.98 ^a	0.98 ^a	0.98

a,b,c ; Means with different superscripts on the same row are significantly different (P<0.05)

SEM; Standard Error of Means

Birds fed AGP had high final weight, weight gain though not significantly ($P>0.05$) different from birds fed *Bacillus subtilis*, feed intake was also higher for birds on AGP and differ significantly ($P<0.05$) across the treatment. The high performance of the AGP showed that antibiotics improve feed efficiency and growth rate. This could be attributed to its antimicrobial effect especially in handling the pathogenic species. Chlortetracycline, oxytetracycline and penicillin have been found to improve growth rate when supplemented in animal feed (Anonymous, 2015). Broiler chicks fed diet supplemented with 225,000 cfu *Bacillus subtilis* shows high performance in terms of final weight, weight gained, best and least feed cost per kg gain (N 235.2) and feed conversion ratio (1.60). *Bacillus subtilis* at 225,000 cfu /100kg feed, reduced

feed intake, increased body weight gain and also improved feed conversion of broiler chicks better than other levels. This level of inclusion equally significantly decreased the cost of production, thereby resulting to higher profits for the farmer. The result of this work agrees with the report of Mohnl, (2011) who reported that direct fed microbial (DFM) supplementation promotes favorable condition in the intestine for the colonization of beneficial microflora, which in turn facilitated better growth performance of broiler chicks. Table 2 shows the performance characteristics of broiler chickens fed levels of *Bacillus Subtilis*. The result shows non-significant ($P>0.05$) difference in weight gain, whereas significant ($P<0.05$) differences were observed for final weight, feed intake, feed conversion and feed cost per kilogram gain.

Table 2. Effect of *Bacillus Subtilis* on the performance of broiler chickens 29-49 days

Parameter	0	75,000 cfu	150,000 cfu	225,000cfu	oxytetracycline	SEM
Initial weight (g/b)	1004.90 ^b	1065.50 ^a	1063.20 ^a	1074.70 ^a	1079.00 ^a	27.25
Final weight (g/b)	2386.80 ^b	2595.60 ^a	2444.70 ^a	2475.00 ^a	2539.80 ^a	87.13
Weight gain (g/b)	1381.90	1530.10	1381.50	1400.30	1460.70	79.73
Feed intake (g/b)	2727.30 ^b	2738.70 ^b	2722.70 ^b	2660.70 ^b	3125.30 ^a	81.77
Feed conversion ratio	2.00 ^b	1.80 ^a	2.00 ^b	1.90 ^{ab}	2.10 ^b	0.07
Feed cost (₦/Kg)	145.20	145.40	145.70	145.70	152.50	0
Feed cost/ gain (₦/ Kg gain)	286.90 ^b	261.30 ^a	288.60 ^b	278.10 ^{ab}	327.10 ^b	10.32
Mortality	1.90	0.90	2.00	2.00	0.99	1.26

a,b, ;Means with different superscripts on the same row are significantly different ($P<0.05$)

SEM;Standard Error of Means

Broiler chickens fed diet supplemented with 75,000 cfu showed best performance in terms of final weight, feed consumed, feed conversion and least cost of production. This level of inclusion is lower than the 150,000cfu/100kg feed recommended by the manufacturers. This is contrary to the result of the starter chicks where levels higher than manufacturers' recommendation showed significant improvement in growth parameters measured. The result of this work agrees with the report of Falaki *et al.* (2011) who reported that improvement in growth

performance and feed efficiency in broilers chickens fed probiotic may be attributed to the total effect of probiotic action including the maintenance of beneficial microbial population (Fuller, 1989), improving feed intake and digestion and altering bacterial metabolism.

Table 3 shows the haematological indices of broiler chicks fed levels of *Bacillus Subtilis*. All parameters measured were not significantly ($P>0.05$) influenced by the dietary treatment. All parameters measured fell within the normal range for healthy chickens as reported Mitruka and Rawnsley

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(1977), and Jain, (1986). This is an indication that the birds were adequately nourished, not anaemic and that the dietary protein is of good quality (Schalm *et al.*, 1975). The blood is often time used as a physiological index or indicator for disease diagnosis, nutrition status and health

condition of the birds. Olafedehan *et al.* (2010) reported that blood acts as a pathological reflector of the status of exposed animals to disease and other conditions. Isaac *et al.* (2013) reported that animals with good blood composition were likely to show good performance.

Table 3. Haematological Indices of Broiler chicks fed levels of *Bacillus Subtilis*

Parameters	0	75,000 cfu	150,000 cfu	225,000cfu	Oxytetracycline	SEM
PCV (%)	42.20	37.90	37.00	35.70	34.80	4.80
Hb (mg/ dL)	12.50	11.30	10.20	10.90	10.40	1.37
WBC (x10 ⁹ /L)	89.60	101.60	82.50	84.50	75.00	10.33
RBC (x10 ¹² /L)	2.80	2.60	2.30	2.52	2.40	0.33
Heterophils (%)	2.60	2.65	2.70	3.80	2.67	0.63
Lymphocytes (%)	90.90	94.20	92.80	88.40	92.00	1.37
MCV (fl)	148.50	147.90	151.40	148.10	145.70	1.76
MCH (pg)	44.10	43.90	45.90	43.50	43.60	1.30
MCHC (g/dL)	29.80	29.70	30.30	30.60	29.90	0.73

SEM;Standard Error of Means

Table 4 shows the results of liver function test of broiler chicks fed levels of *Bacillus subtilis*. Significant (P<0.05) difference were observed in Aspartate amino transferase (AST) Alkaline Phosphatase (ALP) and Albumin. The amount of the enzymes AST and ALP decreased significantly with Birds fed levels of

Bacillus subtilis. The inclusion of *Bacillus subtilis* resulted into a decrease in the amounts of these enzymes which were produced by the liver and the amounts present in the blood is indicative of the integrity of the liver. It is reported that if ALP and AST are found together in elevated amounts in the blood, liver damage is most likely present (WebMed, 2016).

Table 4 Liver function indices of broiler chicks fed levels of *Bacillus Subtilis*

Parameters	0	75,000 cfu	150,000 cfu	225,000 cfu	Oxytetracycline	SEM
AST (μL)	56.00 ^a	43.30 ^a	40.30 ^a	33.30 ^b	45.30 ^b	8.79
ALT (μL)	5.30	4.70	6.00	5.00	6.00	1.23
ALP (μL)	85.80 ^a	83.67 ^b	72.30 ^b	76.70 ^b	91.20 ^a	2.72
TP (g/dl)	2.40	2.30	2.20	2.60	2.10	0.17
ALB (g/ dL)	1.10 ^b	1.30 ^b	1.40 ^a	1.80 ^a	1.30 ^b	0.20
GLB (g/dL)	1.40	1.05	0.78	0.77	0.77	0.23

^{ab}Means with different superscripts on the same row are significantly different (P<0.05): Aspartate amino transferase (AST) Alkaline Phosphatase (ALP) : Alanine amino transferase (ALT); Albumin (ALB); Globulin (GLB). SEM: Standard Error of Means

Thus, the reduced amount of these liver enzymes in the blood of birds fed levels of *Bacillus subtilis* may indicate an improvement in the health of the liver and showed that the material does not impair the liver in its function. Such impairment or damage to the liver would have caused a

leakage of these enzymes, leading to high levels in the blood. Also the reduced level of ALP indicated that the food used in this work provided high digestible protein as high value of ALP is an indication of poor quality protein (Ologhobo *et al.*, 1993). Serum albumin is a strong predictor of

health; a low albumin concentration is a sign of poor health and predictor of bad outcome (Kastow, 2009). The higher the value of albumin the higher the clotting ability of blood, hence prevention of haemorrhage thus this material (*Bacillus Subtilis*) has no toxic or damage-causing effect on the liver.

Table 5 shows the results of the effect of *Bacillus subtilis* on villi morphometrics of section of the ileum. There was significant ($P<0.05$) differences in all parameter measured except for villi width. Villi area, height, width, crypt depth and villi height/crypt depth ratio were higher for birds fed levels of *Bacillus Subtilis* and AGP (Oxyteracycline) than the control.

Table 5: Villi morphometrics of broiler chicks fed levels of *Bacillus Subtilis*

Parameter	0	75,000 cfu	150,000 cfu	225,000 cfu	oxytetracycline	SEM
Area (μm^2)	4678.6 ^c	6252.1 ^{ab}	5112.3 ^b	6485.0 ^a	6564.2 ^a	139.48
Perimeter (μm)	1148.8 ^b	1594.2 ^a	1439.3 ^a	1604.3 ^a	1699.3 ^a	142.03
Villi height (μm)	473.6 ^b	696.8 ^a	485.6 ^b	705.5 ^a	769.4 ^a	63.55
Villi width (μm)	199.7	202.4	203.6	211.2	223.6	16.96
Crypt depth (μm)	113.5 ^b	159.4 ^a	148.9 ^a	160.5 ^a	160.7 ^a	14.03
Villi height/crypt (μm)	3.17 ^b	4.41 ^b	3.93 ^b	4.59 ^b	6.78 ^a	0.26

a,b,c, ;Means with different superscripts on the same row are significantly different ($P<0.05$)

SEM;Standard Error of Means

This results agrees with the findings of Sherief *et al.* (2011) who reported that longer villi were noticed in birds fed Bro-bio-fair® a probiotic containing *Saccharomyces cerevisiae*. it also consistently increase the crypt depth of the mucosa of the small intestine resulting in better FCR for birds fed Bro-bio-fair®. in this work also, the significantly better or higher villi height/crypt ratio translates or amount to a better FCR and higher body weight gain for birds that were fed *Bacillus Subtilis* containing diets. The morphology of intestinal villi and crypts has been associated with intestinal function and growth in chickens. Higher intestinal villi are associated with increased absorptive surface area of the intestine and thus, an increased absorptive capacity with resultant higher body weight gain (Kanduri *et al.*, 2013). A lower villus/crypt ratio has been associated with the presence of toxins,

poor nutrient absorption and increased secretion in the gastrointestinal tract, diarrhea, reduced disease resistance and lower overall performance. A large crypt indicates a fast tissue turnover and a high demand for new tissue (Xu *et al.*, 2003). The result obtained from this work indicates that the gut morphology may be responsible for the growth performance of the broiler chickens.

Table 6 shows the results of the effect of *Bacillus subtilis* on caecal bacteria. There was no significant ($P<0.05$) differences in all parameter measured. However, numerically, the levels of *Bacillus subtilis* in the diet increased beneficial bacteria population such as *Bacillus spp* and *Lactobacilli spp* and also prevent the colonization of the gut by pathogenic bacteria like *Clostridium perfringens*, *Salmonella spp* and *Escherichia coli spp*.

Table 6. Effect of *Bacillus subtilis* on caecal Bacteria species

Parameters	0	75,000 cfu	150,000 cfu	225,000 cfu	oxytetracycline	SEM
<i>Escherichia coli spp</i>	18.00	8.00	5.67	4.67	1.00	6.38
<i>Salmonella spp</i>	13.33	10.67	4.00	3.00	0.00	5.02
<i>Clostridium spp</i>	2.00	1.33	0.67	0.00	1.00	0.76
<i>Bacillus spp</i>	17.00	26.33	20.33	24.67	21.33	9.83
<i>Lactobacilli spp</i>	9.00	12.00	14.67	18.67	0.67	6.59

SEM: Standard Error of Means

Probiotics have been reported to prevent the colonization of the gut by pathogenic bacteria like *Clostridium perfringens* and *Salmonella* through the mechanism of competitive exclusion (Teo and Tan, 2007). A *Bacillus subtilis*-based probiotic is capable of producing an antimicrobial factor against many bacteria (Yurong *et al.*, 2005). Probiotic supplementation plays a crucial role in countering enteric bacterial infections, especially inhibiting pathogens such as *Staphylococcus aureus*, *Escherichia coli*, *Salmonella enteritidis*, *S. typhimurium*, *Clostridium perfringens*, *Listeria monocytogenes*, *Campylobacter jejuni*, *Yersinia enterocolitica*, *Candida albicans* and the coccidian parasites

Eimeria sp. By reducing the intestinal pathogenic microbial load, probiotics lower the pathogen spread in the poultry house via fecal contamination (Dhama *et al.* 2011). Other reports showed that a probiotic based on *Bacillus Cereus* var. *toyoi* reduced the prevalence of *Salmonella* in poultry (Vilà *et al.*, 2009).

Table 7 shows the results of the total antioxidant capacity of broiler chickens fed levels of *Bacillus Subtilis*. There were no Significant ($P > 0.05$) difference in all parameters measured except for Glutathione peroxidase (GPx). GPx activity was significantly ($P < 0.05$) higher for birds fed levels of *Bacillus Subtilis* when compared with the control and AGP.

Table 7. Total Antioxidant capacity of broiler chickens fed levels of *Bacillus subtilis*

Parameters	0	75,000 cfu	150,000 cfu	225,000 cfu	oxytetracycline	SEM
MDA	1.33	1.50	1.60	1.70	1.50	0.20
SOD	2.30	2.20	1.80	1.80	2.00	0.36
CAT	44.00	47.00	42.00	41.00	45.00	3.01
GPX	40.33 ^b	44.00 ^a	48.00 ^a	45.00 ^a	40.00 ^b	3.03

a,b, ; Means with different superscripts on the same row are significantly different ($P < 0.05$)

SEM; Standard Error of Means; Glutathione peroxidase (GPx); Superoxide dismutase (SOD); Catalase (CAT); malondialdehyde (MDA)

The increase in activity of glutathione peroxidase may be attributed to the age, colonization resistance, and susceptibility to environmental pathogens of the birds. GPx is the most crucial among all antioxidant enzymes in most cells due to its capacity to regenerate oxidized antioxidants, and reduce hydrogen peroxide to water and lipid peroxides to their respective alcohols. The GPx is a selenium-dependent enzyme, which decomposes peroxides using the peptide glutathione (GSH) as its co-substrate (Halliwell, 2006). SOD catalyzes dismutation of superoxide radicals to hydrogen-peroxide and oxygen; CAT catalyzes the breakdown of hydrogen-peroxide to water and molecular oxygen. The MDA content in body indirectly reflects the lipid peroxidation, which is the consequence of the attenuated antioxidant protection when reactive oxygen species

(ROS) increase (Yang *et al.*, 2008). Nutrition plays a huge role in maintaining the pro-oxidant-antioxidant balance (Cowe, 1986). An excessive production of reactive species (RS) causes an imbalance in the pro-oxidant/anti-oxidant systems, and any imbalance in favor of the pro-oxidants potentially leading to damage is termed oxidative stress. Oxidative stress is a common process in life, and it could produce varieties of ROS in body, such as hydroxyl free radicals and superoxide anions. Numerous studies showed that excess of ROS can damage the proteins, nucleic acids, and other biological macromolecules, and produce large amounts of MDA leading to tissue damage, thus contributing to the development of diseases. The lipid content is relatively high in broiler chickens, which tends to damage the body through producing ROS for its

nutritional and physiological characteristics. Presently, much attention has been focused on new approaches of anti-oxidant therapy by providing anti-oxidant enzymes. All aerobic organisms possess antioxidant enzymes capable of preventing membrane cell damage, enzyme inactivation and nucleic acid alterations. Excess ROS in body is removed by the antioxidant enzymes, including SOD, GSH, GR, GPx and CAT (Ko *et al.*, 2004). The effect of *Bacillus subtilis* on the Immune response of broilers is presented in Figure1. The results show that the level of

albumin to globulin ratio increased for birds fed levels of *Bacillus subtilis* and AGP than in the control after each vaccination of Gumboro and Lasota. Globulin level has been used as indicator of immune responses and source of antibody production. Griminger, (1986) stated that high globulin and Abumin/globulin ratio (A/G) levels signify better disease resistance and immune response. Noverr and Huffnagle (2004) indicated that some probiotic could stimulate a protective immune response sufficiently to enhance resistance to microbial pathogens.

Fig. 1 Effect of *Bacillus subtilis* on Birds' Immune response to Vaccinations

The results indicated that supplementing *Bacillus Subtilis* may improve immune response This result is in agreement with the result of Kabir *et al.* (2005) who evaluated the dynamics of probiotics on immune response of broilers and reported significantly higher antibody production ($P<0.01$) in experimental birds as compared to control ones. Probiotics supplementation enhances disease resistance and improved the immune response of broiler chickens. A study by Haghighi *et al.*, (2006) suggested that probiotics have an immune-boosting role due to enhanced induction of natural

antibodies in chickens.

Conclusion and Application

Supplementing broiler diet 225,000 cfu and 75,000 *Bacillus Subtilis*/100 Kg feed for starter and finisher chickens, respectively significantly improved birds performance and lowers cost of production. *Bacillus subtilis* supplementation enhanced disease resistance and improved the immune response of broiler chickens. *Bacillus subtilis* supplementation had no adverse effect on heamatological and liver function parameters measured. *Bacillus subtilis*

supplementation significantly improved gut morphology and thus resulting to the improved birds' performance. *Bacillus subtilis* can potentially replace antibiotic growth promoters-AGP.

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