Evaluation of *Bacillus subtilis* spores probiotic as an antibiotic alternative to protect broiler chickens against pathogenic *E.coli* and *Clostridum perfringens*

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1. Abstract

This study was conducted to evaluate the efficacy of *Bacillus subtilis* spores probiotic as an alternative to antibiotic growth promoter by monitoring its effect on broiler chickens performance and determination of the intestinal bacterial count. Three experimental trials were conducted on 480 broiler chickens, 160 birds per each one, four treatments (40 birds) with four replicates (10 broilers per pen). In the first experimental trial; T1: control group (supplemented with basal diet) was compared against T2: supplemented with basel diet + amoxicillin 50% as an antibiotic growth promoter, T3: supplemented with basal diet + *Bacillus subtilis* spores probiotic and T4: supplemented with basal diet + both antibiotic and probiotic together. An improvement was recorded in feed conversion ratio (FCR) on 42-days-old broiler chickens supplemented with *Bacillus subtilis* spores probiotic (T3) when compared with the other groups. Reduction in *E.coli* count in T2 and T3 was observed. Notable reduction in *clostridium perfringens* count in T3 compared to others. Treatments of the second and third experiment were: T1: unmedicated, unchallenged group: control, T2: unmedicated, challenged group, T3: medicated with amoxicillin 50%, challenged group, T4: medicated with *Bacillus subtillis* spores probiotic, challenged group, challenge was done with *E.coli* and *Clostridium perfringens* in the second and third experiment respectively. The results demonstrated a reduction in *E.coli* and *Clostridium perfringens* count in treatment supplemented with *Bacillus subtilis* spores probiotic (T4) and also show a better FCR in both experiments. The current study concluded that the *Bacillus subtilis* spores probiotic could replace antibiotic growth promoter under investigation with better FCR and lower intestinal bacterial pathogens count.

Keywords: broilers; probiotic; amoxicillin; *E.coli*; *Clostridum perfriengens*

2. Introduction

During the past 50 years, different generations of antibiotics have been used in poultry market as therapeutic agents to treat bacterial infections. Many of these antibiotics classes have been used as therapeutic agents for human as well. The usage of these antibiotics has developed hazards concerning human health, such as, development of some bacterial resistance genes to some antibiotics.

Shortly, an increasing debate regarding the use of antibiotics in poultry farms worldwide has been developed due to the concerns over transfer of antibiotic resistance genes, residues in food products.

In Europe, [1] has reported for the first time that there was a relation between growth promoting antibiotics usage in animals and

antibiotic resistance in humans.

In June of 1999 the European Union (EU), banned the use of some growth promoting antibiotics in poultry feeds [2]. These antibiotics are tylosin, spiramycin, bacitracin, viringiniamycin, carbadox and olaquindox. Remaining growth promotion uses were banned in 2006. [3] and [4]. The ban was because of the critical observations that prospect human pathogens, persistently found on processed poultry, were resistant to some antibiotics.

After that ban, there was a dramatic influence in broilers farms. So, there is a great need to identify new alternatives to replace the role of AGP in broilers farms. Now, many alternatives are under study, among these alternatives is probiotics.

Probiotics in animal feed has many useful effects including the competitive exclusion of some pathogenic bacteria. It also promotes the growth and viability of beneficial bacteria in GIT.

Probiotics may increase the population of beneficial micro-organism including *lacto-bacilli* and *bifidobacteria* which then inhibit growth of harmful micro-organisms by producing inhibiting substances (bacteriocins and/or organic acids) and by competitive exclusion. [5].

Looking for new additives able to improve gut health has become even more important with the suppression of antibiotic growth promoters. [6]

The aim of the present study is to evaluate the effect of *Bacillus subtilis* spores probiotic on broiler chickens and demonstrating its ability to be a good alternative to antibiotic growth promoters in poultry production.

3. Materials and methods

3.1. Experimental Design

A total of 480 one-day old un-sexed broilers (Ross 308) were equally divided into three groups 160 birds per each. Each group was divided into four treatments with four replicates, 10 birds per pen.

Treatments of the first group were T1: control (hasn't been supplemented by antibiotic growth promoter or probiotics in its diet) T2: supplemented with Amoxicillin 50%, T3: supplemented with *Bacillus subtilis* spores probiotic, T4: supplemented with Amoxicillin 50%+ *Bacillus subtilis* spores probiotic).

Experiment on the first group tested the effect of *Bacillus subtilis* based probiotic and amoxicillin 50% to prevent some enteric pathogenic bacteria (*E.coli* and *Clostridium perfringens)* in broilers.

Treatments of the second and third group are T1: unmedicated, unchallenged group, T2: unmedicated, challenged group, T3: medicated with amoxicillin 50%, challenged group, T4: medicated with *Bacillus subtillis* spores probiotic, challenged group.

Experiment on the second and third group investigated the effect of *Bacillus subtilis* based probiotic on growth performance and intestinal bacterial count of broilers under *E.Coli* and *Clostridium perfringens* challenges respectively.

3.2. Diet formulation

Both probiotic (*Bacillus subtilis*) and antibiotic (amoxicillin 50%) powder were added to the control diet of the tested group as follows:

Control diet (corn/soybean meal based diet) table (1)

Control diet + *Bacillus subtilis* spores probiotic (inclusion rate: 500g/1000 kg of feed)
Control diet + Amoxicillin 50% (inclusion rate: 400g/1000 kg of feed)
Control diet + *Bacillus subtilis* spores probiotic (inclusion rate: 500g/1000 kg of feed) + Amoxicillin 50% (inclusion rate: 400g/1000 kg of feed)

Table 1: Composition of the experimental starter, grower, and finisher diets.

Ingredient	Starter (1–10 days)	Grower (11–25 days)	Finisher (26–35 days)
	Control	Control	Control
Yellow corn	507	548	578
Soybean meal, 46%	370	317	280
Corn gluten meal, 60%	38	50	50
Soya oil	37	41	51
Calcium carbonate	14.0	13.8	12.6
Di-calcium phosphate	20.0	17.5	16.0
Salt	2.3	2.4	2.3
Sodium sulfate	1.8	1.6	1.6
DlMethionine,99%	2.7	2.0	1.9
l-Lysine HCl, 98%	2.5	2.3	2.2
l-Threonine	1.1	0.7	0.6
Choline chloride, 60%	0.8	0.8	0.8
Premix *	2	2	2
Anti-mycotoxin biology	0.5	0.5	0.5
Silica	1	1	1

Chemical Analysis on DM basis

Ingredient	Starter (1–10 days	Grower (11–25 days)	Finisher (26–35 days)
	Control	Control	Control
AME kcal	3000	3100	3200
Crude protein, %	23.0	21.5	20.0
Fat, %	6.3	6.9	7.9
Digestible LYS, %	1.28	1.15	1.06
Digestible M and C, %	0.95	0.87	0.83
Digestible THR, %	0.86	0.77	0.71
Digestible ARG, %	1.37	1.25	1.14
Digestible ILE, %	0.90	0.85	0.77
Digestible LEU, %	1.87	1.84	1.74
Digestible VAL, %	0.96	0.91	0.84
Calcium, %	0.96	0.87	0.81
Available P, %	0.48	0.44	0.41
Sodium, %	0.16	0.16	0.16
Chloride, %	0.23	0.23	0.23

* Multi Mix® (Multi Vita, Cairo, Egypt). Composition (per 2 kg): Vitamin A 12,000,000 IU, vitamin D3 2,200,000 IU, vitamin E 10,000 mg, vitamin K3 2000 mg, vitamin B1 1000 mg, vitamin B2 5000 mg, vitamin B6 1500 mg, vitamin B12 10 mg, niacin 30,000 mg, biotin 50 mg, folic acid 1000 mg, pantothenic acid 10,000 mg, manganese 60,000 mg, zinc 50,000 mg, iron 30,000 mg, copper 4000 mg, iodine 1000 mg, selenium 100 mg, and cobalt 100 mg. Diets ingredients and final feed diets were analyzed by chemical analysis in the AGROLAB LUFA, GmbH.

3.3. Antibacterial sensitivity test

Five antibiotic discs (Oxoid Ltd,UK) were used in *E.coli* antibiotic sensitivity test (Amoxicillin: 25 µg / Ampicillin: 10 µg / Gentamicin: 30 µg / Norfloxacin: 5 µg / Tetracycline: 30 µg).

Four antibiotic discs (Oxoid Ltd,UK)were used in *Clostridium perfringens* antibiotic sensitivity test (Amoxicillin: 25 µg / Ampicillin: 10 µg / Erythromycin: 15 µg / Lincomycin: 15 µg).

Based on the zones of inhibition's diameters for all selected antibacterial discs, Amoxicillin was the most efficient antibiotic that gives suitable zone of inhibition and it is also considered one of the most effective antibiotic used for *E.coli* and *Clostridium perfringens* infections.

3.4. E. coli Challenge procedure

According to [7] On day 7, from T2 to T4 birds groups were orally challenged with 1.0 mL (2×10^8 cfu/mL) of the freshly grown *E. coli K88* inoculants using a 1-mL pipette (Eppendof, Hamburg, Germany), while the control group, T1 was administrated with the same volume of saline solution. On d 14, and 28, four birds per treatment were randomly selected, euthanized, and intestinal mucosa samples collected.

3.5. Coccidian and Clostridium perfringens Challenge procedure

3.5.1. Coccidian Challenge method

Coccidial challenge was done 3 days before the *Clostradium perfringens* challenge On day 16, 10 folds of recommended vaccination doses of attenuated *coccidial* vaccine (paracox-8) have been orally inoculated to birds. [8]

3.5.2. Clostradium perfringens Challenge methods

The challenge strain (A 15-h FTG broth culture inoculated with a 3% (v/v) overnight CMM stock and incubated at 37°C which contains 10^8 *Clostradium perfringens*/mL) was presented to birds through feed, inoculated at a ratio of 1.25-1.5 fluid FTG: feed (v/w). Birds were starved overnight to eat the first batch of infected feed. Contaminated feed was fed to birds for 4 days (19,20,21,22). Four birds from each treatment were euthanized on the day 23 (the first day after the last challenge) and intestinal samples were collected.

3.6. Enumeration and Identification of E. coli and Clostridium perfringens

The ascending portion of the duodenal loop, the Ilium portion of the GIT and part of the jejunum surrounding the Meckel's Diverticulum were immediately placed on ice till processing.

The duodenal and ileum portions were rinsed with 10 mls of sterile peptone buffer, the jejunum portion was rinsed with 5mls of sterile peptone buffer, and the three sections were squeezed out of any remaining contents. Each section was longitudinally cut to expose the mucosal surface.

3.7. Isolation and identification of E. coli:

Isolation and identification of *E. coli* was done by conventional method: According to [9]. Samples were inoculated into buffer peptone water and incubated at 37˚C for 18 -24 hours under aerobic

condition. A loopful from the enriched samples was streaked onto selective differential media (MacConkey's agar and Eosin Methylene blue agar). Then, the plates were incubated aerobically at 37°C for 24 hours. The suspected colonies were examined for their colonial morphology then examined microscopically and identified biochemically.

On MacConkey's agar, the circular, moist, smooth, bright pink with entire margins colonies were supposed to be *E. coli* colonies, while On Eosin Methylene blue agar (EMB): the obtained green metallic sheen colonies were supposed to be *E. coli* colonies.

Colonies were identified by Gram's stain as Gram negative medium sized bacilli. The isolates were examined by different biochemical tests including Indole reaction (+ve), Methyl red test (+ve), Voges Proskauer test (-ve), Citrate utilization test (-ve), Oxidase test (-ve), Triple sugar iron (acid / acid with gas production) and Christener's urea agar test (-ve).

The API 20 E system tests were done according to the manufacturer instructions and interpretation was done according to API system (Bromers)

3.8. Isolation of Clostridium perfringens:

Samples were diluted in normal saline (1:10), the temperature was maintained at 80°C for 10 minuets to eliminate the non-spore-forming bacteria. [10]

Then, they were cultivated on T.S.C (Ttryptose sulfite cycloserin) medium and incubated anaerobically by Gas pak at 37C˚for 48hrs. And on TSN agar (Tryptone Sulfite Neomycin) (SIGMAALDRICH, USA), the medium was inoculated with the samples and incubated at 46 ± 1°C for 24 hrs. by anaerobic jar (Oxoid Anaerobic Jar). [11]and [12]

The obtained typical colonies were transferred into sheep blood agar and incubated anaerobically at 37°C for 48 hours.

3.9. Identification of Clostridium perfringens:

According to [13] the suspected colonies were examined for their colonial morphology then examined microscopically and identified biochemically. The obtained typical colonies with double zone of Beta hemolysis were supposed to be *Clostridium perfringens*. A bacterial film was done using the suspected colonies and Gram stain has been applied. Spore forming bacilli were observed.

The obtained typical colonies were identified by different biochemical tests such as motility test (non-motile), fermentation of sugars like glucose, lactose and mannitol (ferment glucose, lactose and lactose with acid production) and (lecithinase) (high lecithinase activity) test on egg yolk salt agar.

4. Results and Discussion

The major objective of the current study was to evaluate the efficacy of *Bacillus subtilis* spores probiotic as an alternative to antibiotic growth promoter and its impact on broiler chicken˙s performance and intestinal bacterial count.

The inclusion of *Bacillus subtilis* spores probiotic in broilers diets in the present study improved the growth performance in broilers including feed conversion ratio (table 2) and reduced the intestinal pathogenic bacterial count (table 3 and 4). These findings are in correspondence with [14] demonstrated the efficacy of *Bacillus subtilis* PB6, on broiler

performance as it is acid and heat stable and can produce antimicrobial like substance, which lowers the GIT pathogens count.

The current study reported that the broilers supplemented with *B. subtilis* spores probiotics demonstrated a worthy weight gain but less than the treatment supplemented with antibiotic growth promotor (table 2), dissimilar to [14]**,** who reported a great increase in body weight gain in the broilers supplemented with *B. subtilis* spores probiotics. A higher improvement in FCR was observed in broiler chickens supplemented with *B. subtilis* spores probiotics treatment compared with other treatments.

Necrotic enteritis caused by *Clostridium perfringens* and Avian colibacillosis caused by pathogenic *E. coli* are considered from the most significant diseases that cause great losses in poultry industry according to [15]**.** Colibacillisis is a widespread disease, which is responsible for severe economic losses in the world's poultry industries. [16].

Our results demonstrated reduction in intestinal bacterial count in birds infected with *E.coli.* in T1 and T4 on day 14, and T3 and T4 on day 28 (table 6) and better FCR in T4 (table 5). Also, reduction in intestinal bacterial count in birds infected with *clostridium perfringens* was observed (table 7) and improved FCR (table 8). Moreover, [17] reported that using of probiotics help for the prevention of pathogenic intestinal Enterobacteriaceae and improve the body performance even the chicken infected and added observations of an increase in the immunity of the chicks.

Additionally, [18] stated that reduced *E.coli* counts were seen with multi strain Bacillus spp. Probiotic compared to negative control birds.

In accordance with [2]**,** the present study concluded that *B. subtilis* could act as an economic and safe alternative for antimicrobial growth promoters in broilers production industry.

Similar to our results, [5] stated that probiotics could be a potential alternative to antibiotic feed additives to manage the enteric pathogen load in poultry, by reducing intestinal colonization of common zoonotic and other enteric pathogens.

Recently, [19] concluded that dietary addition of probiotic bacillus spp. Strains affect body weight and intestinal morphology thought altering intestinal microbiota composition in broilers chickens

Experiment 1
Performance results:

Table (2): Body weight gain (kg/bird), feed intake (kg/bird), FCR and production efficiency index

	Body weight gain (Kg/bird)	**Feed intake (kg/bird)**	**FCR**	**European Production Efficacy Factor**
T1	2.16	4.19	1.94	238.6
T2	2.33	4.25	1.82	259
T 3	2.28	4.19	1.81	284.9
T4	2.08	4.26	1.86	239.6

Treatment 1: control, Treatment 2: amoxicillin 50%, Treatment 3: *Bacillus subtilis*, Treatment 4: amoxicillin 50% + *Bacillus subtilis*.

Isolation of *E. coli* from the examined samples:

Table (3): The effect of dietary supplementation of *Bacillus subtilis* spores probiotic on total aerobic bacterial count (CFU/g)

E. coli count / 4 birds / each treatment / from week 2 to week 5				
	Week 2 (CFU/g)	**Week 3 (CFU/g)**	**Week 4 (CFU/g)**	**Week5 (CFU/g)**
T1	$1.4*10^{4.3}$	$2.0*10^{4.3}$	$1.0*10^{3}$	$0.7*10^{4}$
T2	$0.8*10^{3}$	$0.9*10^{4}$	$1.4*10^{4}$	--
T3	$0.6*10^{3}$	$1.1*10^{4}$	$0.8*10^{4}$	--
T4	$1.1*10^{2.3}$	$1.4*10^{4}$	$1.1*10^{4.3}$	$1.2*10^{3}$

Treatment 1: control, Treatment 2: amoxicillin 50%, Treatment 3: *Bacillus subtilis*, Treatment 4: amoxicillin 50% + *Bacillus subtilis.*

Isolation of *Clostridium perfringens* from the examined samples:

The effect of dietary supplementation of *Bacillus subtilis* on total aerobic bacterial count (CFU/g) in samples collected from 64 broilers; (4 birds/treatment/week) is summarized in table (4)

Table (4): The effect of dietary supplementation of *Bacillus subtilis* spores probiotic on total aerobic bacterial count (CFU/g)

Clostridium perfringens count / 4 birds / each treatment / from week 2 to week 5				
	Week 2 (CFU/g)	**Week 3 (CFU/g)**	**Week 4 (CFU/g)**	**Week5 (CFU/g)**
T 1	0	0	$1.1*10^{2.6}$	$0.9*10^{3.3}$
T 2	0	0	$0.9*10^{2.3}$	$0.7*10^{3.3}$
T 3	0	0	$0.6*10^{1.3}$	--
T 4	--	0	$0.7*10^{2.3}$	$1.0*10^{2.6}$

Treatment 1: control, Treatment 2: amoxicillin 50%, Treatment 3: *Bacillus subtilis*, Treatment 4: amoxicillin 50%+ *Bacillus subtilis*.

Experiment 2
Performance results:

Table (5): Body weight gain (kg/bird), feed intake (kg/bird), FCR

	Body weight gain (Kg/bird)	**Feed intake (kg/bird)**	**FCR**
T 1	1.33	2.41	1.81
T 2	1.27	2.66	2.1
T 3	1.39	2.52	1.82
T 4	1.34	2.41	1.80

Treatment 1: control, Treatment 2: challenged, Treatment 3: amoxicillin 50%, challenged, Treatment 4: *Bacillus subtillis* spores probiotic, challenged.

Table (6): Intestinal bacterial count in broilers on 14 and 28 d of age

	Treatment			
	T1	**T2**	**T3**	**T4**
Count at 14 d	**Mean (log_{10} CFU/g)**			
E.coli	6.7	7.8	7	6.9
Count at 28 d				
E.coli	7.4	7.7	7.1	7.2

Measurements were based on 4 broilers per treatment

Experiment (3)
Performance results:

Table (7): Body weight gain (kg/bird), feed intake (kg/bird), FCR

	Body weight gain (Kg/bird)	**Feed intake (kg/bird)**	**FCR**
T 1	1.34	2.49	1.85
T 2	1.31	2.61	1.99
T 3	1.38	2.57	1.86
T 4	1.34	2.45	1.83

Treatment 1: control, Treatment 2: challenged, Treatment 3: amoxicillin 50%, challenged, Treatment 4: *Bacillus subtillis* spores probiotic, challenged.

Table (8): Intestinal bacterial count in broilers on 23 d of age

	Treatment			
	T1	**T2**	**T3**	**T4**
	Mean (log_{10} CFU/g)			
C. Perfringens	4.7	6.0	3.8	3.3

Measurements were based on 4 broilers per treatment

5. Conclusion

B. subtilis spores probiotics could act as an economic and safe alternative for antimicrobial growth promoters Amoxicillin 50% against pathogenic *E.coli* and *Clostridum perfringens* in broilers production industry.

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