Animal Nutrition 1 (2015) 203–212

Contents lists available at ScienceDirect

Animal Nutrition

journal homepage: www.www.keaipublishing.com/en/journals/aninu

Original research article

Use of *Lactobacillus johnsonii* in broilers challenged with *Salmonella sofia*

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ARTICLE INFO

Article history:
Received 6 May 2015
Accepted 24 July 2015
Available online 22 August 2015

Keywords:
Probiotic
Broiler
Challenge
Salmonella sofia

ABSTRACT

The effects of *Lactobacillus johnsonii (L. johnsonii)* on gut microflora, bird performance and intestinal development were assessed using 288 one-day-old Cobb broilers challenged with *Salmonella sofia* (*S. sofia*). The experiment was a 3 × 2 factorial design which consisted of three treatments, a negative control (NC) with no additives, a positive control (PC) containing antimicrobials (zinc-bacitracin, 50 mg/kg) and a probiotic group (Pro), and with the two factors being unchallenged or challenged with *S. sofia*. A probiotic preparation of *L. johnsonii* (10^9 cfu/chick) was administered to chicks individually by oral gavage on days 1, 3, 7 and 12. Chicks were individually challenged with *S. sofia* (10^7 cfu/chick) by oral gavage on d 2, 8 and 13. Results showed that the challenge itself markedly reduced ($P < 0.05$) bird performance and feed intake. And, transient clinical symptoms of the infection with *S. sofia* were observed from the second time they were challenged with *S. sofia* in the negative challenge groups. The novel probiotic candidate *L. johnsonii* reduced the number of *S. sofia* and *Clostridium perfringens* in the gut environment, and improved the birds' colonization resistance to *S. sofia*.

1. Introduction

Probiotics may alter gut microflora in poultry and play a role in competitive exclusion (CE) of *Salmonella* by the Nurmi concept (Pivnick and Nurmi, 1982). Competitive exclusion involves oral administration of intestinal microflora derived from healthy salmonella-free adult birds into newly hatched chicks. Establishment of an adult intestinal microflora in newly hatched chicks increases their resistance to colonization by non-host-specific salmonellae.

The use of CE microflora against *Salmonella* colonization in poultry is proven to be effective (Blankenship et al., 1993; Jin et al., 1998; Gusils et al., 2003). The most important advantage is that CE products ensure the establishment of a complex intestinal microflora that resists colonization by poultry pathogens, and they are produced as a consortium of bacteria that can coexist as a stable community in the enteric ecosystem (Wagner, 2006). Another factor in the use of lactobacilli to induce CE of *Salmonella* is that the members of the *Lactobacillus* family readily utilize lactose in their metabolism. Mannose and lactose may act to inhibit *Salmonella* attachment via different mechanisms; mannose may interact with mannose-sensitive type-1 fimbrae on the bacterium, lactose on the enhancement of the growth of *Lactobacillus*, which, in turn, inhibits the growth of pathogens such as *Salmonella* (Oyofo et al., 1989). The antibacterial effect of Lactobacilli *in vitro* against *Escherichia coli* and *Salmonella* spp. and the bactericidal effect on *Salmonella faecalis* have been documented (Fuller and Brooker, 1974). The results of Pascual et al. (1999) showed that using the rifampin-resistant *L. salvarius* CTC2197 (feed additional concentration as 10^5 cfu/gram) prevents *Salmonella enteritidis* in chickens, and that the pathogen was completely removed from the birds after 21 days.

Salmonella sofia (S. sofia) first came to the attention of the Australian Salmonella Reference Centre in 1979 as a new isolate from chickens. Despite the widespread colonization of chickens by *S. sofia*, it is not represented in the list of serovars isolated from humans, which indicates that it may be of low virulence to humans (Harrington et al., 1991). *Salmonella sofia* is ubiquitous amongst Australian chicken flocks but few serious *Salmonella* food poisoning outbreaks attributed to chicken meat have occurred. In the years 1982 to 1984, *S. sofia* represented approximately 30% of all salmonella isolations from raw chickens in Australia and isolation from chickens rose to a peak of 49% of all isolates in 1988 (Harrington et al., 1991).

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Peer review under responsibility of Chinese Association of Animal Science and Veterinary Medicine.

Production and Hosting by Elsevier on behalf of KeAi

http://dx.doi.org/10.1016/j.aninu.2015.07.001

Chickens are known to be very sensitive to *Salmonella* infections during the first week of life because of delayed development of their intestinal flora. The gastrointestinal tract (GIT) of chickens harbours a microfloral load which is formed immediately after hatching. The mature indigenous microflora forms an important barrier against colonization of potentially pathogenic bacteria, such as *Salmonella* (Fuller, 1997). The microflora of the intestinal tract consists of many different species of microorganisms, *Lactobacillus*, *Bifidobacterium* and *Bacteroides* species being the most predominant groups of microorganisms present in healthy chickens; these constitute about 90% of the flora. Ewing and Cole (1994) reported that the development of the intestinal microbiota commences soon after birth, and the establishment of 'climax conditions' takes days or weeks depending on environmental conditions. During this process, the composition of the microbiota continuously changes as one group of microbes becomes numerically dominant, only to be supplanted by a new group of organisms, which, in turn, is supplanted. In young chicks, administration of gut microflora has been shown to be effective against several *Salmonella* species, such as *Salmonella typhimurium* (Mead, 2000) and *Salmonella kedougou* (Ferreira et al., 2003). The importance of bacterial metabolites and intestinal microflora composition in controlling pathogenic bacterial infections has been well documented in animal models (Hume et al., 1998; Bielke et al., 2003). Literature data suggest the importance of early establishment of beneficial bacterial populations in preventing *Salmonella* colonization using animal models. Based on these principles, a novel probiotic of chicken origin, *Lactobacillus johnsonii*, was selected for this experiment because of its production of bacteriocin-like inhibitory activities that may be effective in controlling *S. sofia* infection in broilers.

2. Materials and methods

2.1. Growing the probiotic strain

The bacterial strain used in this experiment was selected using the antagonistic activity assay described by Teo and Tan (2005).

A pure *L. Johnsonii* isolate was grown in De Man, Rogosa, Sharpe broth (MRS broth) overnight at 39°C and harvested by centrifugation at 4,420 × *g* for 15 min (Induction Drive Centrifugation, Beckman Model J2-21M, Beckman Instruments Inc., Palo Alto, California, USA). It was re-suspended in phosphate-buffered saline (PBS) (pH 7.4) and mixed by constant mechanical stirring (Heidolph MR 3001K stirrer, Heidolph Instruments GmbH & Co., Schwabach, Germany) for 10 min. This pre-mixture of PBS solution was used for oral gavage of chicks. The quantities of MRS broth and pre-mix PBS solution were calculated by the bacterial concentration needed for the experiment. In this study, the concentration of the probiotic candidate *L. johnsonii* was $> 1.28 \times 10^9$ cfu/mL of BPS solution without bacterial extracellular products.

Each chick in the probiotic treatment group was orally administered 0.5 mL of the highly concentrated culture solution using a crop needle on d 1, and 1 mL on d 3, 7 and 12. Birds in other groups received the same amount of sterile PBS solution on the same day.

2.2. Infectious strain of Salmonella sofia

The strain of *S. sofia* was obtained from the Biotechnology Laboratory, RMIT University (Melbourne, VIC, Australia) and maintained in Luria Bertani (LB) broth with 30% (vol/vol) glycerol at −20°C. The strain was made rifampicin resistant as described by Eisenstadt et al. (1994) with some modifications as follows: 1) the gradient plate technique used antibiotic agar containing rifampicin (95% HPLC, R3501-5G, Sigma–Aldrich, Castle Hill, NSW, Australia) at 80 μg/mL; and 2) to more accurately determine the level of resistance to rifampicin, the mutants were each streaked on several plates containing different concentrations of rifampicin, namely, 100, 110 and 120 μg/mL.

The mutant strain was amplified by growth overnight at 39°C in 1,000 mL of LB broth, it was then harvested by centrifugation at 5,000 × *g* for 15 min (Induction Drive Centrifugation, Beckman Model J2-21M, Beckman Instruments Inc., Palo Alto, California, USA), re-suspended in 100 mL (200 mL from second time) of PBS (pH 7.4) to a smaller final volume to produce a highly concentrated culture without bacterial extracellular products. The re-suspended solution was mixed by constant mechanical stirring (Heidolph MR 3001K stirrer, Heidolph Instruments GmbH & Co., Schwabach, Germany) for 15 min. This challenge pre-mixture of PBS bacterium solution was administered by oral gavage.

2.3. Experimental diets and bird husbandry

A total of 288 one-day-old male Cobb broiler chickens vaccinated against Marek's disease, infectious bronchitis, and Newcastle disease were obtained from a local hatchery (Baiada hatchery, Kootingal, NSW, Australia) and assigned to six dietary treatments, each with six replicates, 8 chickens per replicate. Chickens were reared in multi-tiered brooder cages placed in a climate-controlled room. The basal diets (starter and finisher) were based on corn, wheat and soybean meal as shown in (Table 1) and provided as pellets. The six treatments included in this trial were: 1) negative control (NC−), non-probiotic and unchallenged with *S. sofia*; 2) positive control (PC−), as feed additional zinc-bacitracin (50 mg/kg) provided, non-probiotic and unchallenged with *S. sofia*; 3) probiotic control (Pro−), as probiotic inoculated and unchallenged with *S. sofia*; 4) negative challenged (NC+), as non-probiotic, non-antibiotic and challenged with *S. sofia*; 5) positive challenged (PC+), as non-probiotic inoculated, feed additional zinc-bacitracin (50 mg/kg) provided and challenged with *S. sofia*; and 6) probiotic challenged (Pro+), as probiotic inoculated and challenged with *S. sofia*.

Each of the six dietary treatments was divided into two groups, unchallenged and challenged, and randomly assigned to 6 cages for each treatment with 8 birds per cage in each large group. The birds were transferred to slide-in cages in an environmentally controlled room at the end of the third week in the same separation groups. The room temperature was gradually decreased from 33°C on d 1 to 24 ± 1°C at d 35. Eighteen hours of lighting were provided per day throughout the duration of the experiment, apart from d 1 to 7 when 23 h of lighting were provided. Feed and water were provided ad libitum and bird performance was measured on a weekly basis by recording the group weight and feed intake for each cage. Mortalities were recorded as they occurred, and feed per gain values were corrected for mortality.

2.4. Salmonella sofia challenge model

The probiotic inoculation with *L. johnsonii* and the dosage were previously described in Section 2.1.

The infection dose rate of *S. sofia* was 10^7 cfu/mL. This follows the challenge models for salmonella described by Bjerrum et al. (2003). The bacterial suspension was individually administered using a crop needle and a 1-mL syringe with a flexible tube attached. In one series of experiments, chicks were given 0.5 mL of the bacterial suspension on first challenge. On d 8 and 13, chicks were given 1 mL of bacterial suspension. The control groups received correspondingly the same volume of sterile PBS solution. Unchallenged birds were always serviced first to reduce the likelihood of cross-contamination and all inoculation was completed inside the cages.

The climate-controlled rooms were divided into two separate areas to avoid cross infection between the challenged and unchallenged treatments. Treatments were allocated randomly from unchallenged or challenged treatments.

Table 1
Ingredients and calculated chemical composition of basal diets, as-fed basis.

Item	wk 1 to 3 (Starter)	wk 4 to 6 (Finisher)
Ingredient, g/kg		
Wheat	262.0	214.0
Sorghum	350.25	400.2
Mung beans	100.0	100.0
Tallow in mixer	32.5	34.0
Sunflower meal	–	25.0
Canola meal	60.0	60.0
Cottonseed meal	–	50.0
Soybean meal	157.0	81.5
Limestone B10	15.5	16.0
Kynofos/Biofos MDCP	11.5	11.0
Salt	1.75	1.5
Sodium bicarbonate	2.0	2.0
Choline Chloride 75%	0.6	0.6
DL-Methionine	2.1	1.3
L-Lysine scale 3	2.1	0.4
L-Threonine	0.2	–
Vitamin and mineral premix[1]	2.5	2.5
Calculated chemical composition, g/kg		
ME, MJ/kg	12.26	12.39
Crude protein	200.02	190.00
Crude fibre	35.17	43.14
Crude fat	52.16	54.47
Lys	11.49	8.98
Met + Cys	8.32	7.37
Ca	9.73	9.79
Available phosphorous	6.50	6.71
Na	1.62	1.65
Cl	2.19	1.75

[1] Vitamin and mineral premix (Ridley Agriproducts Pty Ltd., Tamworth, NSW) contained the following minerals per kilogramme of diet: vitamin A (as *all-trans* retinol), 12,000 IU; cholecalciferol, 3,500 IU; vitamin E (as D-α-tocopherol), 44.7 IU; vitamin B_{12}, 0.2 mg; biotin, 0.1 mg; niacin, 50 mg; vitamin K_3, 2 mg; pantothenic acid, 12 mg; folic acid, 2 mg; thiamine, 2 mg; riboflavin, 6 mg; pyridoxine hydrochloride, 5 mg; D-calcium pantothenate, 12 mg; Mn, 80 mg; Fe, 60 mg; Cu, 8 mg; I, 1 mg; Co, 0.3 mg; and Mo, 1 mg.

2.5. Sample collection and processing

On d 14 and 35, two birds from each cage were randomly selected and killed by cervical dislocation. The abdominal cavity was opened and visceral organs were weighed. The weight of the full small intestine and then the empty weight of each intestinal segment were recorded.

The contents of the gizzard, ileum and caeca were collected in plastic containers, and stored at −20°C until VFA analysis was performed. A 2-cm piece of the proximal ileum was flushed with ice-cold PBS at pH 7.4 and fixed in 10% formalin for gut morphological measurements. One gram (approximately) each of ileal and caecal fresh digesta was transferred individually into 15 mL MacCartney bottles containing 10 mL of anaerobic broth for bacterial enumeration. An approximately 2 cm piece of the proximal ileum was flushed with ice-cold PBS at pH 7.4 and fixed in 10% formalin for morphological measurements.

Extra ManConkey (Oxoid, CM 0007) agar with rifampicin (80 μg/mL) was used for detecting the number of *S. Sofia*.

To avoid cross infection, samples from the unchallenged treatments were collected first. The challenged treatments were collected after the unchallenged sample collection had been completed. To screen for salmonella, approximately 1 g of spleen, liver, ileum and caecum were placed individually in pre-enriched buffered peptone water (BPW, Oxoid, CM0509) using the process described by Bjerrum et al. (2003). A tenfold dilution series was made in BPW; thereafter 100 μL was streaked on each of three types of agar plates, namely, Rambach ager (Rambach agar, CHROMagar RR701, Dutec Diagnostics, Croydon, NSW, Australia), Luria Bertani (LB) agar [Tryptone (1% wt/vol), yeast extract (0.5% wt/vol), NaCl (0.5% wt/vol)] and bacteriological agar (0.6 to 0.9% wt/vol, dissolved in deionized water), and MacConkey agar with rifampicin (80 μg/mL). Agar plates were incubated aerobically at 39°C for 24 h. For the control groups, extra Rambach agar without rifampicin was used. Colonies were counted after 24 h; the detection limit was 10^2 cfu.

2.6. Digesta pH measurement, VFA analysis and gut histomorphology

Immediately following slaughter, fresh digesta samples weighing about 0.5 g from the gizzard, ileum and caecum were transferred into 15 mL containers and 4.5 mL of distilled water was added and mixed. The pH value of the suspension was determined by the modified procedure of Corrier et al. (1990).

After thawing at room temperature, the concentrations of short-chain fatty acids (SCFA) and lactic acid of each digesta sample from the ileum and caeca were measured using gas chromatography (Varian CP-3800. Netherlands) according to the method described by Jensen et al. (1995).

Tissue samples were collected from the proximal ileum and flushed with buffered saline and fixed in 10% neutral buffered formalin for histomorphological analysis. Samples were embedded in paraffin wax, sectioned and stained with haematoxylin and eosin. Sample sections were captured at 10 × magnification using a Leica DM LB microscope (Leica Microscope GmbH, Wetzlar, Germany) and morphometric indices were determined as described by Iji et al. (2001). Each sample was measured in 15 vertically,

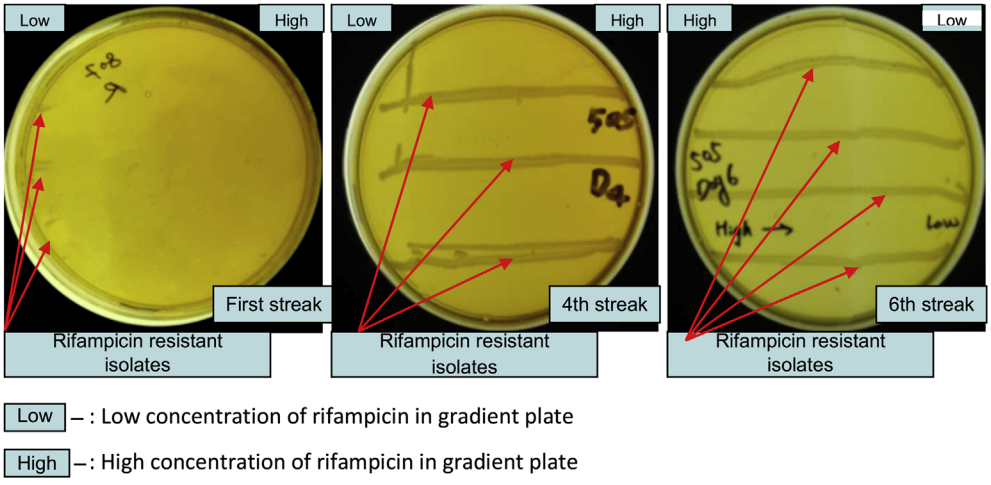

Fig. 1. Preparation of rifampicin resistant isolates of *Salmonella sofia*.

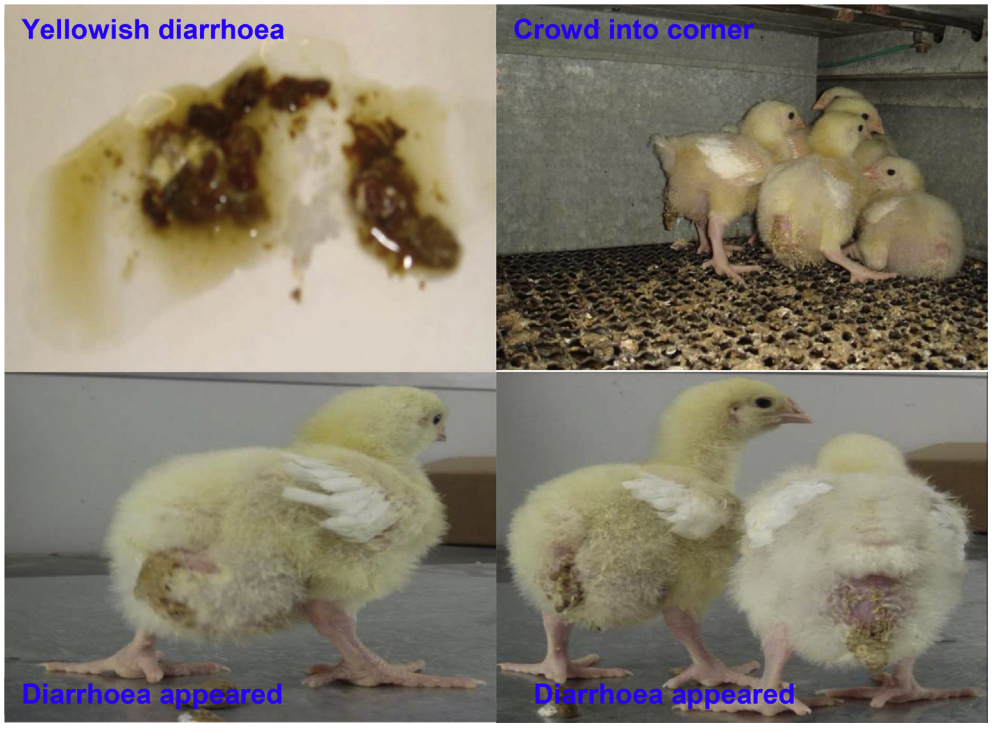

Fig. 2. Symptoms in challenge groups (NC+).

well-oriented, intact villi, muscle depth and crypts photomicrographs of a stage micrometre recorded at 5 × magnification.

2.7. Statistical analysis and animal ethics

Statistical analyses were performed using Statgraphics Plus (Professional Edition, Manugistics Inc., Rockville, Maryland, USA). The data were analysed using multifactor analysis of variance (ANOVA) with treatment and challenge as factors. The differences between means were identified by the least significant difference (LSD). Differences among treatments and challenge were deemed to be significant only if the *P*-value was less than 0.05. Bacterial counts were transformed to $\log_{10}$ values before analysis.

Health and animal husbandry practices complied with the 'Australian code of the care of animals for scientific purposes' issued by the Australian Government National Health and Medical Research Council (NHMRC, 2004). The Animal Ethics Committee of the University of New England approved the experiments in this study (authority number: AEC07/148).

3. Results

3.1. Mutant isolation of Salmonella sofia

The isolates of *S. Sofia* started to grow after the first streak on the side of the mutant gradient plate where the rifampicin

Table 2
Performance[1] of broilers either non challenged or challenged with *S. sofia* on d 14 and 35.

Item	Treatments[2]						*P*-value		
	NC−	NC+	PC−	PC+	Pro−	Pro+	T[3]	C[4]	T × C[5]
d 1 to 7									
BWG, g/bird	169.2	167.6	174.0	169.2	175.7	168.5	0.54	0.67	0.87
FI, g/bird	187.9	189.1	190.1	187.6	196.8	186.5	0.82	0.12	0.51
FCR, g/g	1.11	1.13	1.09	1.11	1.12	1.11	0.24	0.76	0.44
d 1 to 14									
BWG, g/bird	385.1[a]	334.2[b]	401.9[a]	380.8[a]	390.2[a]	377.0[a]	0.03	0.01	0.01
FI, g/bird	462.1[a]	310.0[b]	478.2[a]	453.1[a]	464.4[a]	456.1[a]	0.02	0.01	0.02
FCR, g/g	1.20[a]	0.93[b]	1.19[a]	1.19[a]	1.19[a]	1.21[a]	0.03	0.02	0.04
d 1 to 35									
BWG, g/bird	1,806.8	1,813.5	1,834.6	1,799.7	1,824.5	1,811.7	0.31	0.27	0.17
FI, g/bird	3,112.3	3,234.5	3,129.8	3,079.9	3,154.4	3,189.1	0.94	0.68	0.55
FCR, g/g	1.72	1.78	1.71	171	1.73	1.76	0.59	0.18	0.38
Mortality, %	6.25	8.33	4.17	4.17	6.25	4.17	–		

[a,b] Means within the same row with no common superscript differ significantly ($P < 0.05$).

[1] Values are means ($n = 6$).

[2] Treatments: NC−, unchallenged negative control; NC+, challenged negative control; PC−, unchallenged positive control; PC+, challenged positive control; Pro−, unchallenged probiotic control; Pro+, challenged probiotic control.

[3] T: treatments.

[4] C: challenge.

[5] T × C: variance interaction between treatment and challenge.

Table 3
Effects of relative organ weights[1] (% body weight) of broilers either non-challenged or challenged with S. sofia on d 14 and 35.

Item	Treatments[2]						*P*-value		
	NC−	NC+	PC−	PC+	Pro−	Pro+	T[3]	C[4]	T × C[5]
Day 14									
Liver	4.01	4.04	4.04	4.11	4.04	3.97	0.87	0.98	0.46
Spleen	0.11	0.11	0.11	0.12	0.12	0.11	0.51	0.59	0.71
Pancreas	0.36	0.34	0.39	0.36	0.35	0.39	0.31	0.53	0.84
Bursa	0.24	0.20	0.23	0.22	0.31	0.22	0.23	0.18	0.47
Gizzard	3.52[b]	4.06[a]	3.55[b]	3.41[b]	3.88[b]	4.09[a]	0.02	0.06	0.04
Duodenum	1.53[c]	1.93[a]	1.69[b]	1.89[a]	1.46[b]	1.91[a]	0.01	0.48	0.10
Small intestine	7.28[b]	9.06[a]	6.59[b]	8.52[a]	7.36[b]	8.10[a]	0.01	0.02	0.02
Day 35									
Liver	2.59	2.50	2.66	2.64	2.18	2.71	0.53	0.90	0.48
Spleen	0.09	0.09	0.11	0.11	0.11	0.09	0.25	0.61	0.91
Pancreas	0.15	0.18	0.20	0.18	0.16	0.17	0.88	0.24	0.55
Bursa	0.12	0.15	0.17	0.12	0.13	0.18	0.52	0.63	0.84
Gizzard	2.09	1.56	1.58	1.44	1.30	1.65	0.36	0.69	0.59
Duodenum	0.41	0.48	0.46	0.51	0.43	0.58	0.72	0.34	0.22
Small intestine	4.00	3.59	3.90	4.07	3.85	4.13	0.52	0.18	0.17

[a,b] Means within the same row with no common superscript differ significantly ($P < 0.05$).

[1] Values are means ($n = 6$).

[2] Treatments: NC−, unchallenged negative control; NC+, challenged negative control; PC−, unchallenged positive control; PC+, challenged positive control; Pro−, unchallenged probiotic control; Pro+, challenged probiotic control.

[3] T: treatments.

[4] C: challenge.

[5] T × C: variance interaction between treatment and challenge.

Table 4
Digesta pH and short chain fatty acid concentrations[1] (μmol/g) on birds either non challenged or challenged with *S. sofia* on d 14 and 35.

Item	Treatments[2]						*P*-value		
	NC−	NC+	PC−	PC+	Pro−	Pro+	T[3]	C[4]	T × C[5]
Day 14									
Gizzard									
pH	2.95	2.61	3.40	3.09	3.31	2.98	0.27	0.43	0.56
Ileum									
pH	6.24	5.91	6.44	6.22	6.01	6.42	0.51	0.23	0.47
Formic acid	0.42	0.46	0.37	0.31	0.49	0.55	0.19	0.57	0.72
Acetic acid	2.37[a]	1.59[b]	2.46[a]	1.74[b]	2.49[a]	1.85[b]	0.02	0.01	0.01
Lactic acid	9.32	10.23	10.47	10.73	9.81	10.67	0.63	0.55	0.82
Caeca									
pH	5.89	5.91	5.63	5.54	5.79	6.17	0.19	0.61	0.25
Acetic acid	47.21[a]	31.42[b]	45.61[a]	30.41[b]	49.54[a]	34.29[b]	0.03	0.01	0.09
Propionic acid	3.46	3.14	2.97	3.51	3.16	3.29	0.47	0.33	0.46
Butyric acid	15.42	15.71	15.29	14.83	15.66	15.09	0.32	0.87	0.89
Total VFA	83.42[a]	64.71[b]	80.21[a]	61.64[b]	84.17[a]	63.72[b]	0.12	0.01	0.14
Day 35									
Gizzard									
pH	2.85	3.05	2.94	2.76	3.11	3.28	0.23	0.75	0.51
Ileum									
pH	7.78	7.56	7.53	7.55	7.39	7.26	0.17	0.29	0.47
Formic acid	1.24	1.02	0.97	1.19	1.11	1.29	0.31	0.79	0.84
Acetic acid	2.67	2.55	2.37	2.46	2.48	2.69	0.52	0.27	0.61
Lactic acid	–	–	–	–	–	–	–		–
Caeca									
pH	5.52	5.63	5.46	5.48	5.29	5.36	0.11	0.46	0.39
Acetic acid	74.32	75.19	72.64	76.21	72.18	69.94	0.71	0.59	0.45
Propionic acid	3.98	4.51	4.33	3.81	3.49	4.09	0.82	0.38	0.70
Butyric acid	13.84	14.27	13.56	13.94	13.72	14.17	0.57	0.22	0.38
Total VFA	97.21	101.24	98.81	98.67	95.76	96.48	0.42	0.58	0.71

[a,b] Means within the same row with no common superscript differ significantly ($P < 0.05$).

[1] Values are means ($n = 6$).

[2] Treatments: NC−, unchallenged negative control; NC+, challenged negative control; PC−, unchallenged positive control; PC+, challenged positive control; Pro−, unchallenged probiotic control; Pro+, challenged probiotic control.

[3] T: treatments.

[4] C: challenge.

[5] T × C: variance interaction between treatment and challenge.

Table 5
Effects of experimental treatment[1] on bacterial counts (lg cfu/g) in digesta of birds either non-challenged or challenged with *S. sofia* on d 14 and 35.

Item	Treatments[2]						*P*-value		
	NC−	NC+	PC−	PC+	Pro−	Pro+	T[3]	C[4]	T × C[5]
Day 14									
Ileum									
Total anaerobes	7.61	7.48	7.14	7.35	8.08	8.09	0.36	0.51	0.62
LAB	7.31	7.01	6.96	8.11	7.28	7.84	0.48	0.29	0.61
Lactobacilli	7.24	6.88	6.61	7.97	7.94	7.23	0.52	0.16	0.21
Enterobacteria[6]	5.07^{c}	6.17^{a}	5.19^{c}	6.32^{a}	5.51^{b}	6.45^{a}	0.03	0.01	0.04
C. perfringens	2.96	3.76	3.77	3.76	3.58	3.57	0.15	0.27	0.25
S. sofia[7]	0.00^{c}	6.11^{a}	0.00^{c}	4.78^{b}	0.00^{c}	5.09^{b}	0.01	0.01	0.01
Caeca									
Total anaerobes	9.51	9.42	9.22	9.22	9.43	9.36	0.33	0.54	0.48
LAB	9.07	9.11	9.03	9.16	9.42	9.19	0.78	0.66	0.63
Lactobacilli	8.48	8.58	8.72	8.94	9.12	8.85	0.26	0.33	0.19
Enterobacteria	8.19^{b}	9.07^{a}	8.45^{b}	8.87^{a}	8.55^{b}	8.91^{a}	0.03	0.01	0.03
C. perfringens	6.29^{b}	7.86^{a}	6.14^{b}	7.38^{a}	5.99^{b}	8.15^{a}	0.01	0.01	0.01
S. sofia	0.00^{c}	8.97^{a}	0.00^{c}	5.57^{b}	0.00^{c}	5.70^{b}	0.01	0.01	0.01
Day 35									
Ileum									
Total anaerobes	7.55	8.19	7.98	7.88	7.89	8.23	0.15	0.24	0.40
LAB	7.51	7.95	7.86	7.61	7.49	7.72	0.36	0.17	0.20
Lactobacilli	7.05^{b}	7.48^{b}	7.35^{b}	7.38^{b}	8.16^{a}	8.60^{a}	0.04	0.01	0.02
Enterobacteria	5.78	6.74	5.83	6.37	5.93	5.72	0.57	0.28	0.10
C. perfringens	2.90	3.03	2.91	3.15	3.21	2.99	0.27	0.52	0.74
S. sofia	0.00	0.00	0.00	0.00	0.00	0.00	–	–	–
Caeca									
Total anaerobes	8.52	8.55	8.94	8.99	8.76	8.64	0.85	0.57	0.43
LAB	8.77	8.37	8.86	8.92	8.35	8.36	0.29	0.33	0.64
Lactobacilli	7.96^{c}	7.63^{c}	8.50^{b}	8.51^{b}	9.03^{a}	9.30^{a}	0.01	0.01	0.01
Enterobacteria	7.91	7.66	7.13	7.92	7.72	7.27	0.30	0.55	0.29
C. perfringens	5.13^{b}	6.55^{a}	4.17^{c}	6.29^{a}	4.44^{c}	6.27^{a}	0.04	0.01	0.05
S. sofia	0.00	0.00	0.00	0.00	0.00	0.00	–		

LAB = lactic acid bacteria.
$^{a, b, c}$ Means within the same row with no common superscript differ significantly ($P < 0.05$).

[1] Values are means ($n = 6$).
[2] Treatments: NC−, unchallenged negative control; NC+, challenged negative control; PC−, unchallenged positive control; PC+, challenged positive control; Pro−, unchallenged probiotic control; Pro+, challenged probiotic control.
[3] T: treatments.
[4] C: challenge.
[5] T × C: variance interaction between treatment and challenge.
[6] Enterobacteria are coliform and lactose negative enterobacteria.
[7] The detection limit of the cfu was 10^2, samples registered as zero could still contain small amounts of *S. sofia*.

concentration was low (80 μg/mL). After the sixth streak, however, the strain grew strongly, showing resistance to 120 μg/mL of rifampicin on the agar (as shown in Fig. 1). Indeed, results proved that the mutant strain grew normally in LB broth, reaching concentrations of *S. sofia* higher than 2.5×10^7 cfu/mL in BPS solution (data not shown).

3.2. Clinical symptoms of challenged birds and mortality

Clinical symptoms were observed in the birds after the second time they were challenged with *S. sofia* in the NC+ group, but not detected in other treatment groups (Fig. 2). Within a few hours of the second inoculation, chicks were showing obvious clinical symptoms; they huddled in the corners of the cage, showing somnolence, loss of appetite and inhibition in drinking. They were generally depressed and reluctant to move, A thin, yellowish diarrhoea appeared with some chicks. The clinical symptoms were transient, however, and these behavioural changes were pronounced for about 8 h, then disappeared gradually, recovery being complete within 24 h. None of the chicks died during the 48 h after inoculation. The mortality rate for these chickens was less than 8.3% (4/48) compared with the NC group where it reached 6.25% (3/48).

Table 6
Results of enrichments from different organs[1] on birds either non-challenged or challenged with *S. sofia* on d 14 and 35.

Treatments[2]	d 14				d 35			
	Spleen	Liver	Ileum	Caecum	Spleen	Liver	Ileum	Caecum
Control	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12
NC+	12/12	12/12	11/12	12/12	3/12	2/12	0/12	2/12
PC+	12/12	11/12	7/12	12/12	1/12	0/12	0/12	0/12
Pro+	12/12	12/12	6/12	12/12	0/12	1/12	0/12	0/12

[1] The salmonella enrichments were conducted total of 12 birds each treatment and numbers of positive birds showed as in table.
[2] Treatments: Control, means include negative control, positive control and probiotic control; NC+, negative challenge; PC+, positive challenge; Pro+, oral gavage with probiotic and challenged.

Table 7
Ileal morphormetry[1] of broilers either non-challenged or challenged with *S. sofia* on d 14 and 35.

Item	Treatments[2]						*P*-value		
	NC−	NC+	PC−	PC+	Pro−	Pro+	T[3]	C[4]	T × C[5]
Day 14									
Villus height, μm	665	671	674	669	663	671	0.84	0.71	0.92
Crypt depth, μm	91	88	92	94	86	92	0.24	0.53	0.48
Villi:crypt ratio	7.32	7.61	7.36	7.13	7.68	7.29	0.16	0.28	0.42
Muscle depth, μm	310	309	314	317	305	315	0.31	0.77	0.69
Day 35									
Villus height, μm	773	758	770	763	769	775	0.13	0.24	0.53
Crypt depth, μm	127	129	137	122	126	128	0.37	0.37	0.64
Villi:crypt ratio	6.08	5.94	5.87	6.22	6.14	6.07	0.21	0.55	0.71
Muscle depth, μm	411	426	408	414	419	427	0.46	0.18	0.29

[1] Values are means ($n = 6$).
[2] Treatments: NC−, unchallenged negative control; NC+, challenged negative control; PC−, unchallenged positive control; PC+, challenged positive control; Pro−, unchallenged probiotic control; Pro+, challenged probiotic control.
[3] T: treatments.
[4] C: challenge.
[5] T × C: variance interaction between treatment and challenge.

3.3. Gross response

Growth, FI and FCR were all depressed during the second week in NC+ treatment compared with the other treatments. However, this trend was not evident in the following weeks. By the end of the 5-week experimental period there was no difference in performance between the challenged and unchallenged groups (Table 2).

3.4. Organ weights, intestinal pH and SCFA concentrations

The relative weights of the gizzard, duodenum and small intestine were increased in challenged groups compared with unchallenged groups on d 14. No significant change in the weight of any other organ was detected in birds after being challenged with *S. sofia* (Table 3).

The concentration of acetic acid significantly decreased in the challenged group and the lowest concentration was found in the NC+ treatment in both ileal ($P < 0.05$) and caecal ($P < 0.01$) digesta on d 14 (Table 4). This trend was not detected on d 35. There was also no significant difference in the concentration of formic, propionic and butyric acids between the challenged and unchallenged groups on d 14 and 35 in the ileum and caecum. Lactic acid was not detected in the ileal digesta on d 35.

3.5. Bacterial populations in intestinal digesta

No differences in total anaerobes and lactic acid bacteria numbers in the ileal and caecal contents were found between the treatment and control groups (Table 5). The number of *Enterobacteria* found in the ileum and caecum on d 14 was higher in the challenged groups than in the unchallenged groups. The number of *Clostridium perfringens* in the caecal contents of unchallenged groups (NC−, 6.29; PC−, 6.14; Pro−, 5.99) was lower ($P < 0.05$) than those in the challenged groups (NC+, 7.86; PC+, 7.38; Pro+, 8.15) on d 14. This trend was also found on d 35, but the negative control (5.13) was higher ($P < 0.05$) than the positive (4.17) and probiotic (4.44) in unchallenged control groups. Furthermore, the number of lactobacilli was higher ($P < 0.05$) in the probiotic control and probiotic challenged groups on d 35.

The salmonella counts from the ileum and caeca on sampling days are shown at Table 5. Three successive inoculations with 1×10^7 cfu of *S. sofia* established a high level of infection in the ileum and caeca, which was detectable from d 14. Chickens that received a high dose of *S. sofia* inoculation appeared to establish the most stable infection, with the number of salmonella reaching around 6.11 cfu/g in the ileum and 8.97 cfu/g in the caeca. The number of *S. sofia* in the ileal and caecal digesta was significantly ($P < 0.01$) decreased in PC+ and Pro+ groups compared with NC+ treatment on d 14. No *S. sofia* was detected in the digesta from the ileum and caeca on d 35.

At each sampling, chickens were taken out from both the challenge group and control groups. The control chickens were free of *Salmonella* throughout the experiments, verified by LB agar both with or without rifampicin and by enrichments from spleen, liver, ileal digesta and caecal digesta (Table 6). However, by using enrichment it was found that the spleen and liver became positive for salmonella, detected from sampling d 14 for most chickens in challenge groups, but towards the end of the experiment fewer positive samples were found from the organs. It was also shown that the ileum had a low level of salmonella present for most chickens on sampling d 14.

3.6. Intestinal histomorphology

In the ileum, villus height, crypt depth and muscle depth in the challenged treatments did not differ from the control groups (Table 7). In both unchallenged and challenged treatment groups, the villus:crypt ratio ranged from 7.13 to 7.68 (d 14) and 5.87 to 6.22, respectively, not significantly different among treatments.

4. Discussion

4.1. Mutant strain of S. sofia

Genetic and biochemical investigations in bacteriology are often initiated by the isolation of mutants. The power of mutational analysis derives from its ability to query an organism incisively. Rifampicin-resistant mutants can be easily isolated from *S. sofia*. The results indicated that *S. sofia* growing on the mutant gradient plates (80 μg/mL) started at the first streak. The resistant strain grew satisfactorily on agar plates containing 100 or 120 μg/mL of rifampicin after the third streak. This is supported by Bjerrum et al. (2003) who demonstrated that salmonella mutants can grow on agar plate containing higher than 50 μg/mL concentration of the rifampicin.

4.2. Clinical symptoms and bird performance

Older birds inoculated with salmonella parenterally were less easily infected than when they were younger. The symptoms – reluctance to move, depression, somnolence, loss of appetite and inhibition in drinking appeared on d 8 of age, after the second inoculation. However, there were no visible symptoms by d 13. This is in agreement with Rahimi et al. (2007) who reported that clinical

symptoms disappeared two days after administration. Methner et al. (1995) studied the *S. typhimurium* and *S. enteritidis* infection model at different ages of chickens, and their results agree with the present results that the same dose of inoculation can produce different effects at different ages. Bjerrum et al. (2003) have also used different infection doses of *S. typhimurium* on 14-day-old chicks. They showed that an inoculation dose of 10^7 had the optimal invasiveness at 2 weeks of age but no clinical symptoms were observed.

In this experiment, we used an established 1-day-old chick model to assess the effects of *L. johnsonii* upon colonization and persistence of *S. sofia*. Short-term symptoms appeared in the negative challenged group on d 8, but were not observed in other challenged groups. The result indicated that *L. johnsonii* acted against *S. sofia* infection and reduced the clinical symptoms affecting bird performance. Humbert et al. (1991) indicated that bacitracin (50 mg/kg) gave the best protection in salmonella-challenged chickens compared with other antibiotics.

Salmonella sofia is the predominant serovar isolated in Australian chickens and 50 to 60% of salmonella chicken isolates belong to this group (Heuzenroeder et al. (2001). Because *S. sofia* is avirulent and does not cause disease in humans or poultry (Harrington et al., 1991; (Heuzenroeder et al. (2001), very little is known or understood about the clinical symptoms of *S. sofia* infection of chickens. Maybe it is because only high doses ($>10^7$) of infection produce clinical symptoms in chickens.

4.3. Organ weights and concentrations of SCFA

The salmonellosis symptoms were accompanied by a decrease in BWG in the NC+ treatments and this led to relatively heavier gizzard and small intestine in challenged groups at 14 days of age. The duodenum showed a similar trend. These results are in accordance with those of Ivanov (1977) who reported similar clinical symptoms in chicken were treated with lipopolysaccharide in *Salmonella gallinarum* infections.

The concentration of lactic acid from ileal digesta on d 35 was below a detectable level in either challenged or unchallenged treatments. Similar findings were reported by Van der Wielen et al. (2000) from their *in vivo* experiments where they detected lactate during the first 15 days only.

Significant negative correlations were observed between numbers of *Enterobacteria* and acetic acid concentration in the ileum and caeca. The result showed a significantly lower acetic acid concentration in ileal and caecal digesta in the second week of the experiment in the challenged groups when compared with unchallenged groups. Reports concerning correlations between VFA and *Enterobacteria* have mainly focused on the intestines of mice (Pongpech and Hentges, 1989). Furthermore, Van der Wielen et al. (2000) have demonstrated that the decrease in numbers of *Enterobacteria* can lead to increased production of acetate in the caeca of chickens. This appears to be the only study on poultry in the literature, albeit it is of the opposite view. In the current study, with a lower concentration of VFA groups (NC+ and PC+) there were higher numbers of *Enterobacteria* in the ileum (6.17 and 6.32) and caeca (9.07 and 8.87) on d14. This is supported by many studies by Freter and Abrams (1972), Byrne and Dankert (1979), Pongpech and Hentges (1989) in which it was observed that a higher concentration of total VFA is related to a reduced number of *Enterobacteria*. Whether it is related to *Enterobacteria* being highly susceptible to increases in VFA in the gut is not known. In fact, the correlation between VFA concentrations and the number of *Enterobacteria*, and its significance remain speculative.

However, Freter and Abrams (1972) did not observe any relationship between VFA and *Enterobacteria* in mice. The pH values for the caecum of mice in their study ranged from 6.5 to 7.0. At these pH values, the concentrations of VFA are very low. In the present experiment, pH values were around 5.5 to 6.2 in the caeca on d 14. This might explain the significant correlations observed from our results in the caeca of chickens, in contrast to those observed in the caecum of mice.

One of the mechanisms by which the intestinal microflora may reduce *Enterobacteria* is the bacteriostatic effect of VFA in the GIT. This will be discussed in Section 4.4. However, the current study showed that the VFA production is one of the mechanisms responsible for the decrease in numbers of *Enterobacteria* in the ileum and caeca of broilers.

4.4. Gut microfloral populations

Three inoculations with 1×10^7 cfu of *S. sofia* established a high level of infection in the ileum and caeca, which was detectable from samples obtained at d 14. Chickens receiving the same level of high dose of *S. sofia* established the most stable infection in challenged groups, with higher than 6.11 cfu/g concentrations in the GIT.

It was found that the number of *Enterobacteria* in challenged groups was higher than in unchallenged groups in the ileum and caeca on d 14, but not on d 35. However, to use of the rifampicin resistant strain allowed the identification and quantification of the infection strain in intestinal samples. The current result showed in *L. johnsonii* inoculated groups, the number of lactobacilli markedly increased and in the number of *S. sofia* significantly decreased. Furthermore, *C. perfringens* numbers in the caeca were lower (<5.99, <4.44) in the probiotic treatment than in other challenged groups (>7.38, >6.27) on both sampling days. It was documented by La Ragione and Woodward (2003) that a single oral dose of 1×10^9 cfu *L. johnsonii* inhibited the growth of S. *enteritidis* and *C. perfringens* and reduced the extent of colonization and persistence in 1-day-old and 20-day–old chick models. Also Pascual et al. (1999) found rifampicin-resistant *Lactobacillus salivarius* reduced *S. enteritidis in vivo* together with its ability to colonize the gastrointestinal tract of chickens after a single inoculation. This growth inhibition to *S. enteritidis* was also observed by Van der Wielen et al. (2002) who used *Lactobacillus crispatus* in their *in vitro* study.

One of the mechanisms by which the intestinal microflora may reduce *Enterobacteria* is the bacteriostatic effect of VFA in the gastrointestinal tract. It has been demonstrated that *in vitro* supplemental VFA inhibited growth of *Enterobacteria* at pH 6 (van Immerseel et al., 2003). Newly hatched chicks are highly susceptible to salmonella infection (Desmidt et al., 1997). Possibly the acetate content in the caeca of young chickens and the lack of other SCFA add to the susceptibility of these young animals. The probiotic strain *L. johnsonii* may increase the VFA concentration after inoculation. The CE culture was administered to broilers a day before salmonella was administered, resulting in a dramatic reduction in the number of salmonella observed (Van der Wielen et al., 2002). Results obtained in the current study are in agreement with these findings on CE cultures *in vivo*. Watkins and Miller (1983) suggested that *Lactobacilli* spp. increase competitive exclusion against harmful organisms (*S. typhimurium*, *Staphylococcus*, and *E. coli*) in the intestinal tract of chickens.

The gut microflora is the determining factor in the viability of specific microorganisms. The production of VFA at pH below 6.0 is known to decrease the population of *Salmonella* and *Enterobacteria* (Meynell, 1963). Disruption of the normal intestinal microbial population with antibiotics will abolish this mechanism of CE because the concentration of VFA produced by the intestinal bacteria will decrease and gut pH will increase towards a more alkaline range. In newly hatched chicks, the VFA concentration and pH are not sufficient to chemically exclude pathogens (Barnes and Impey, 1980).

Previous results showed that, after oral inoculation, *L. johnsonii* becomes a dominant species in the GIT. The most important advantage is that CE products ensure the establishment of the complex intestinal microflora that resists colonization by poultry pathogens, and they are produced as a consortium of bacteria that

can coexist as a stable community in the enteric ecosystem (Wagner, 2006). The major factor to consider when choosing a CE agent to reduce *Salmonella* is that the *Lactobacillus* family utilize lactose readily in their metabolism. It has pointed out by Oyofo et al. (1989) that mannose and lactose may act to inhibit *Salmonella* attachment via different mechanisms. Mannose may interact with mannose-sensitive type-1 fimbrae on the bacterium. Lactose, on the other hand, known to inhibit the growth of pathogens *in vivo* (Schaible, 1970), may act by the enhancement of the growth of *Lactobacillus*, which, in turn, inhibits the growth of *Salmonella* (Oyofo et al., 1989).

4.5. *Salmonella* enrichment in organs and digesta

From the reports, most salmonella challenge experiments operate with 10^4 to 10^6 cfu/g given orally to small chickens (Baha et al., 1991; Fukata et al., 1991; Ziprin et al., 1993). Also Bjerrum et al. (2003) indicated that dose levels of around 10^7 cfu/g yielded stable infections in 14-day-old chickens. In the current study the spleen and liver of chicks became positive for salmonella on d 14, although only a few remained positive at end of the experiment. In addition, the ileum had the lowest level of salmonella present in most chickens at d 14. This is supported by Bjerrum et al. (2003) who demonstrated that the passage time through the ileum is very fast compared with that of the caeca where the bacteria have more time to establish. Other authors have pointed to the caeca as an important segment of infection as well, the lumen of the caeca being the main site of colonization for salmonella rather than the epithelium (Barrow et al., 1988). They also found long-term infection in the ileum of birds inoculated at d 1, whereas no *Salmonella* could be detected in the ileum of chickens inoculated at d 21. This observation was confirmed in the current study which found no *Salmonella* in the ileum at d 35.

Salmonella could be recovered from the spleen and liver of both challenged groups, and this is supported by results from d 35 in the current study. This experiment did not identify the time period when *Salmonella* was recoverable. Bjerrum et al. (2003) and Barrow et al. (1988) confirmed that the period for recovering *Salmonella* was 1 or 2 d after exposure to *Salmonella*. Hassan et al. (1991) found that infection of the spleen with *S. typhimurium* persisted for about 4 to 5 weeks post-inoculation. Also Bjerrum et al. (2003) indicated that the clearing of the organs is dependent on chicken age rather than time post-inoculation, a finding which was also supported by the work of Methner et al. (1995). Samples were not assessed daily in present experiment, and were therefore only able to confirm *S. sofia* infection in the spleen and liver on d 35.

5. Conclusion

The infection model for *S. sofia* resulted in stable colonization of the ileum and caeca for chickens receiving three successive inoculations starting from d 2. This study demonstrated that oral inoculation with the novel probiotic *L. johnsonii* was able, through CE, to reduce *S. sofia* and *C. perfringens* in GIT, and provide resistance to *S. sofia* in broiler chickens.

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