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Original article

POSITIVE EFFECTS OF DIETARY PROBIOTICS ON IMMUNE RESPONSE AND GUT MORPHOMETRY IN BROILER CHICKENS

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Summary

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An experiment was performed with a total of 280 one-day old SPF broiler chicks to evaluate the effects of probiotics, alone or in combination, on growth performance, gut morphometry and immune response to fowl cholera vaccination. The birds were randomly divided into seven groups each of 40 chicks and the experiment lasted for 42 days. The probiotic microorganisms that were offered via water included *Lactobacillus acidophilus, Bifidobacterium bifidum, Enterococcus faecium*, *Bacillus subtilis* and *Saccharomyces cervisiae*. Significant increase in the food conversion rate was recorded in group 4 that received probiotic mixture composed of *Lactobacillus acidophilus* and *Bacillus subtilis*. Also, significantly high geometric mean titre (GMT) of *P. multocida* specific-antibodies and lowest morbidity and mortality rates post *P. multocida* challenge were recorded in this group. The effect of different probiotics on the morphometric changes in the gut tissues was determined, where significant increase in the duodenal and ileum villus height and average crypt depth were recorded in probiotic treated chicks compared to the negative control. The increase in the gut villi height is proved to be associated with increased absorption capability of nutients from the intestine.

Key words: broiler chickens, growth performance, gut morphometry, immune response, probiotics

INTRODUCTION

Poultry industry is one of the fastest growing agricultural and veterinary sectors. Feed is an important item of expenditure in poultry production accounting for 70% of total costs (Bidarkar *et al.,* 2014). The constant increase in the cost of compound

poultry feed and feed ingredients stand behind the significant yield reduction for poultry farmers. Numerous feed additives like antibiotics have been broadly used as growth promoters for increasing poultry production. However, their use is either banned or associated with development of several health hazards to consumers (Jadhav *et al.*, 2015). The development of antibiotic resistant bacterial strains and residual effects of these feed additives in eggs and meat (McEwen *et al.,* 2018) necessitates the prevention of antibiotics use in many countries (Apata, 2009). Therefore, search of antibiotics alternatives for use as growth promoters in poultry production became an important necessity (Pournazari *et al.*, 2017).

Recent studies have shown that gut microbiota is able to improve inflammatory response and lessen stress-induced behaviours in humans and rodents via regulation of both the microbiota-gut-brain axis and the microbiota-gut-immunity axis (Brandsma *et al.,* 2015; Yano *et al.*, 2015). The progress of the broiler intestinal microbiota begins at hatching. Therefore, the type of microbes supplemented in the initial days of chickens helps in forming the gut microbial community (Rinttilä & Apajalahti, 2013).

The most preferred and effective alternative to antibiotics are probiotics. They play an important role as growth promoters and pathogens inhibitor in poultry industry (Zhang & Kim, 2014). Probiotics have been recorded to improve feed efficiency (Tabidi *et al.,* 2013), growth performance, meat quality (Liu *et al.,* 2012; Yang *et al.,* 2012; Park & Kim, 2014), immune system (Mahrose *et al.,* 2019) and keep a balanced intestinal ecosystem (Sinol *et al.,* 2012). Khaliq & Ebrahimnezhad (2016) concluded that the use of probiotic from 1 to 42 days in diet improved the performance of broiler chicks.

The most popular species of bacteria used in the production of probiotics include *Lactobacillus bulgaricus, L. acidophillus, L. casei, L. helveticus, L. salvarius, L. plantarum, L. faecalis, Enterococcus faecium, Enterobacter faecalis, Bifidobacterium* spp., *Saccharomyces cerevisiae, Streptococcus thermophilus, Toulopsis sphaerica* and other lactobacilli and streptococci (Jadhav *et al.,* 2015). The usage of *Bacillus subtilis* and *Saccharomyces boulardii* could be applied to accelerate digestive enzyme activities, blood profile of broilers and anti-oxidation (Rajput *et al.,* 2013). Yeasts are among the most effective probiotics proved to enhance birds' performance (Reisinger *et al.,* 2012; Yasar & Akincl, 2014; Chen *et al.,* 2016; Yasar *et al.,* 2016).

The present work was performed to evaluate the effect of different probiotics formulations on the feed conversion ratio. Also its effect on immune response to fowl cholera vaccinations and on the overall morbidity and mortality rates of broilers after challenge with *P. multocida* was determined. Moreover the effect of different probiotic formulations on the gut morphometric characters represented by villi height and crypt depth was studied.

MATERIALS AND METHODS

Experimental birds

The study was carried out according to internationally recognised guidelines for animal welfare. A total of 280 one-day old SPF White Lohman broiler chicks were obtained from a grandparent hatchery in Fayoum Governorate and raised in an experimental open-sided poultry house at the Microbiology Department, Faculty of Veterinary Medicine, Cairo University. Water was provided *ad libitum*, but the

feed offered to the experimental birds was measured. The birds were fed the starter diet from day 1 to 14, the grower diet from day 15 to 28, and the finisher diet from day 29 to 43.

The probiotic microbial species and their concentrations are listed in Table 1. These microbial strains were kindly obtained from Department of Microbiology, Faculty of Veterinary Medicine, Cairo University.

The probiotics listed in Table 1 were used alone or in combination. Five types of probiotic formulations were tested in 5 chicken groups, each including 40 chicks (Table 2). The probiotics were offered via water to the broiler chicks from day 1 to day 42 at the concentration given above. The parameters used to evaluate the probiotic effects on broiler chicks performance included body weight gain, feed intake, feed conversion ratio (FCR), immune response to fowl cholera vaccinations and the overall morbidity and mortality after challenge with *P. multocida*. Also, their effect on the morphometry of the gut was determined. The findings were compared with data obtained from negative control (receiving no probiotics) and positive controls fed a commercial probiotic (Proact). The ProAct is a blend of 7 probiotics, namely *Saccharomyces cerevisiae*, *Saccharomyces boulardii*, *Bacillus liquefaciens*, *Bacillus subtilis*, *Bacillus coagulans*, *Lactobacillus acidophilus* and *Lactobacillus bulgaricus*. It was added through drinking water (3 mL/L). In all groups the probiotics formulations were added in drinking water on a daily base and the viability of it was confirmed by cultivation on specific culture media.

All the groups except the negative control group were vaccinated against fowl cholera and the vaccination was performed on days 7 and 14.

Table 1. Probiotics species and concentration used

Composition	Concentration/ liter drinking water
Lactobacillus acidophilus	3.09×10^{10} CFU/L
Bifidobacterium bifidum	3.00×10^{10} CFU/L
Enterococcus faecium	8.85×10^{10} CFU/L
Bacillus subtilis	1.00×10^{9} CFU/L
Saccharomyces cervisiae	7.98×10^{9} CFU/L

Table 2. The probiotic combinations given to the different broiler chickens groups

Group No	Number of probiotic strains used	Probiotic compositions
Group 1	5	*Lactobacillus acidophilus, Bifidobacterium bifidum, Enterococcus faecium*, *Bacillus subtilis, Saccharomyces cervisiae*
Group 2	4	*Lactobacillus acidophilus, Enterococcus faecium*, *Bacillus subtilis, Saccharomyces cervisiae*
Group 3	3	*Lactobacillus acidophilus, Bacillus subtilis, Saccharomyces cervisiae*
Group 4	2	*Lactobacillus acidophilus, Saccharomyces cervisiae*
Group 5	1	*Lactobacillus acidophilus*
Group 6	Positive control	Commercial Proact probiotic
Group 7	Negative control	–

Feed conversion ratio was weekly calculated as the amount of feed (g) consumed to produce 1 g of live weight, as described by Morgan & Lewis (1962): FCR = feed intake/weight gain.

Pasteurella multocida vaccine was purchased from Institute for Animal Vaccine Production, Ministry of Agriculture, Abbasia, Giza, Egypt. The product contains whole broth culture suspension of *Pasteurella multocida* (each dose contains greater than 10^8 CFU prior to inactivation). The vaccine was inactivated by formalin (0.5% final concentration). The vaccination dose was 0.5 mL of vaccine given intramuscularly in the breast muscle.

Five experimental chicks from each group were randomly selected for bleeding (jugular vein) on days 7, 14, 21, 28, 35 and 42. Serum samples were stored at –20 ^{o}C till examined. Indirect haemagglutination assay as described by Gold & Fudenberg (1976) was used to estimate antibody titre developed against *Pasteurella multocida***.** The HA antibody titre of each serum sample was expressed as the reciprocal of its end-point dilution.

Experimental challenge

Pasteurella multocida virulent strain was kindly supplied from Department of Microbiology, Faculty of Veterinary Medicine, Cairo University. On day 35, 10 birds from each experimental group were challenged subcutaneously with a field *Pasteurella multocida* isolate (1 mL of inoculum) and observed for 10 days.

Histological studies

Twenty eight chickens, four from each experimental group were euthanised and specimens from duodenum and ileum were collected and fixed in 10% formal saline, then washed, dehydrated, cleared and embedded in paraffin. As described by Bancroft & Stevens (1996), the paraffin embedded blocks were sectioned at 4–5 µm thickness, stained with haematoxylin and eosin and examined for the morphometric changes in the villi height and crypt depth of the ileum and duodenum (Olympus BX50, Japan).

Statistical analysis

Data obtained from the differently treated groups was compared by analysis of variance (ANOVA). The statistically significant differences among treatment mean values were estimated using the least significance difference test at 5% probability level.

RESULTS

Table 3 demonstrates the effect of different probiotic formulations on the feed conversion rate in broiler chicks. A statistically significant difference in feed conversion rate (FCR) was recorded at 7, 14, 21, 28, 35 and 42 days of feeding. The highest food conversion rate was recorded in group 4 (*L. acidophilus, S. cervisiae*) followed by Group 3 (*L. acidophilus, B. subtilis, S. cervisiae*).

The effect of different probiotics formulations on the GMT of *P. multocida*-specific antibodies developed against *P. multocida* vaccine in broiler chicks is shown in Table 4. The highest GMT was recorded in Groups 3 and 4.

The effect of different probiotic formulations on the mortality and morbidity rates in vaccinated broiler checks post *P. multocida* challenge was recorded. No morbidity or mortality were recorded in immunised broilers supplemented with *L. acidophilus, B. subtilis, and S. cerevisiae* (Group 3) and in those fed on probiotic formulation composed of *L. acidophilus* and *S. cerevisiae* (Group 4).

Table 3. Effect of different probiotic formulations on feed conversion rate (FCR) in broiler chicks

Day	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	P
7	1.9	1.9	2.4	2.4	1.8	2.3	1.8	0.041
14	1.5	1.6	1.9	2.1	1.7	1.9	1.4	0.021
21	1.8	1.8	2.1	2.2	1.8	2	1.7	0.023
28	1.7	1.8	2.4	2.4	1.9	2.2	1.5	0.009
35	1.5	1.5	1.7	1.9	1.7	1.9	1.4	0.003
42	1.4	1.5	2.0	2.1	1.8	1.9	1.3	0.001

*ANOVA test.

Table 4. Effect of different probiotic formulations on the geometric mean titre of *P. multocida*-specific antibodies in vaccinated broiler chicks as measured with haemagglutination test

Day	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	P
7	76±2.21	75±1.82	79±2.15	79±1.55	77±2.11	79±2.63	75±0.99	0.032
14	58±3.32	57±3.53	66±2.84	67±2.92	57±2.85	67±1.99	37±1.24	0.008
21	67±1.09	70±2.71	89±2.05	90±1.98	68±1.74	89±2.02	59±2.40	0.003
28	101±1.18	110±1.43	121±1.22	130±1.61	109±2.13	127±2.06	89±2.51	0.032
35	130±1.41	138±2.8	169±2.77	173±2.47	133±2.07	171±1.85	110±2.62	0.008
42	165±2.17	160±1.92	192±1.34	195±2.01	162±1.79	193±1.52	121±2.35	0.003

*ANOVA test.

The morphometric changes in the gut tissues of broiler chicken groups received different probiotic formulation are presented on Fig. 1–4. Negative control group (Group 7) which was untreated and non-vaccinated, showed average villus height of 518.69 µm and average crypt depth of 146.75 µm in the duodenum (Fig. 1A) and average villus height of 157.16 µm and average crypt depth of 111.6 µm in the ileum (Fig. 1B).

Positive control group (vaccinated chicks fed on diet containing commercial probiotic) showed average villus height of 495.09 µm and average crypt depth of 149.40 µm in the duodenum (Fig. 2A). The respective values in the ileum were 722.18 and 183.73 µm (Fig. 2B).

Broiler chickens from Group 1 that received probiotic formulation composed of 5 microbial agents, namely *L. acidophilus, B. subtilis, E. faecium, S. cerevisiae* and *B. bifidum*, showed that the villus height and crypt depth of the duodenum was increased compared with control. Also the ileal villus height was increased vs the negative control group and decreased compared to the positive control group. The crypt depth was decreased compared with controls.

Group 2, supplemented with probiotic formulation composed of 4 microbial agents (*L. acidophilus, B. subtilis, E. faecium, S. cerevisiae)* showed decreased villus height and crypt depth in the duodenum compared with controls. Also, the villus height and crypt depth of the ileum were increased compared to negative controls and both were decreased vs positive controls.

Group 3 fed on *L. acidophilus, B. subtilis* and *S. cerevisiae)* had lower villus height and crypt depth of the duodenum than controls (Fig. 3A). The villus height and crypt depth of the ileum was increased as compared to negative controls and lower than positive controls (Fig. 3B).

Group 4 in which broiler chicks received a diet with *L. acidophilus* and *S. cerevisiae* showed higher villus height and crypt depth of duodenum compared with both control groups (Fig. 4A). Also, the ileal villus height was increased compared to negative controls but lower than positive controls. The crypt depth was decreased compared to both controls (Fig.

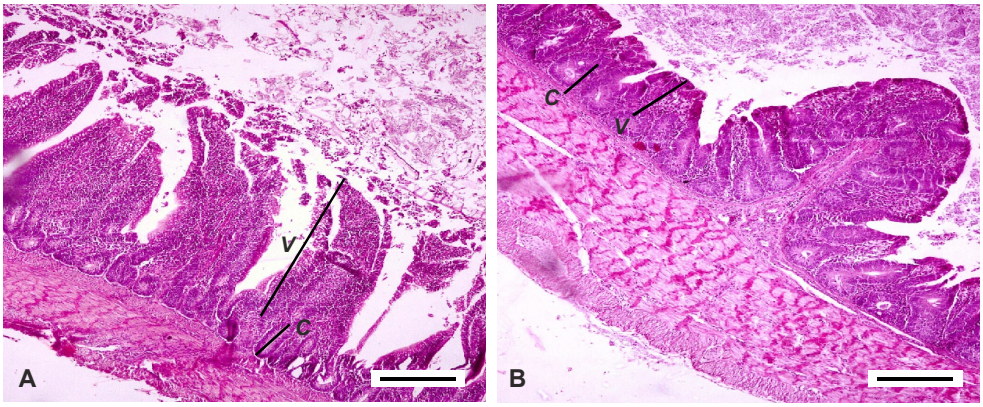

Fig. 1. Photomicrograph of broiler gut from negative control group given no probiotic. **A.** Duodenum with average villus height 518.69 µm (*V*) and average crypt depth of 146.75 µm (*C*). **B.** Ileum with average villus height 157.16 µm (*V*) and average crypt depth of 111.6 µm (*C*). H & E, bar = 155 µm.

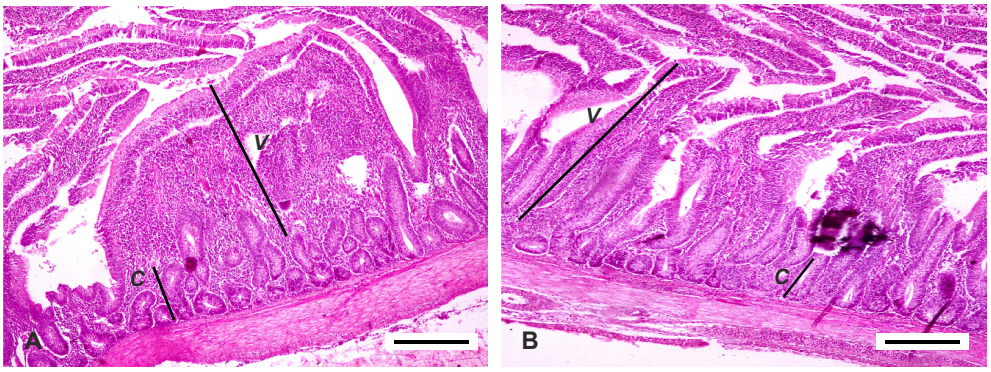

Fig. 2. Photomicrograph of broiler gut from control positive group. **A.** Duodenum with average villus height 495.09 µm (*V*) and average crypt depth of 149.40 µm (*C*). **B.** Ileum with average villus height 722.18 µm (*V*) and average crypt depth of 183.73 µm (*C*). H & E, bar = 155 µm.

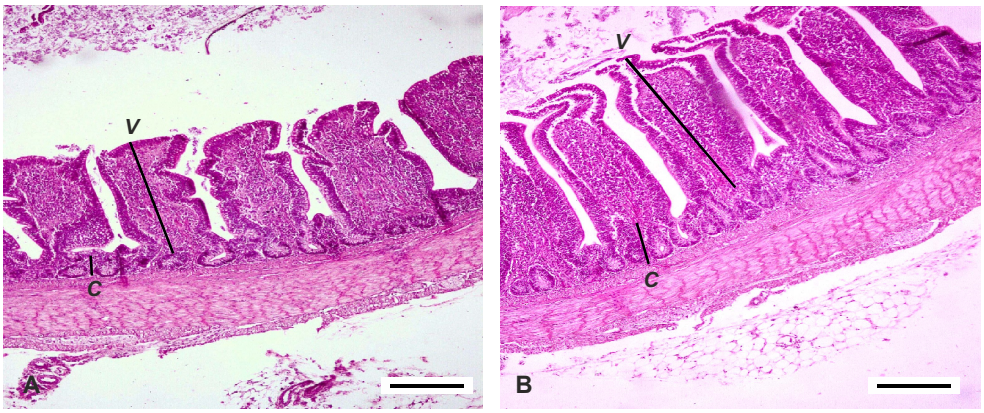

Fig. 3. Photomicrograph of broiler gut from group 3 (*Lactobacillus acidophilus, Bacillus subtilis, Saccharomyces cervisiae*). **A.** Duodenum with average villus height 379.43 µm (*V*) and average crypt depth of 53.33 µm (*C*). **B.** Ileum with average villus height 494.92 µm (*V*) and average crypt depth of 122.04 µm (*C*). H & E, bar = 155 µm.

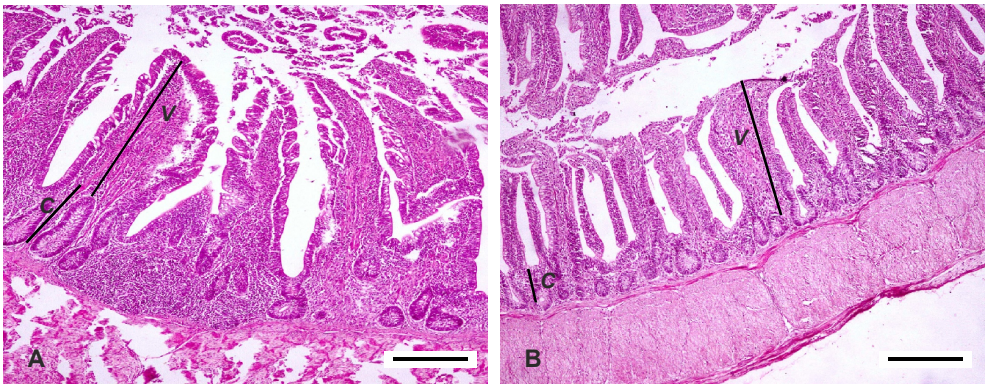

Fig. 4. Photomicrograph of broiler gut from group 4 (*Lactobacillus acidophilus, Saccharomyces cervisiae*). **A.** Duodenum with average villus height 576.79 µm (*V*) and average crypt depth of 208.93 µm (*C*). **B.** Ileum with average villus height 355.57 um (*V*) and average crypt depth of 90.65 um (*C*). H & E, bar = 155 µm.

4B). Similar itestinal morphology changes were found in Group 5, which was supplemented only with *L. acidophilus*.

DISCUSSION

The most preferred and effective alternative to antibiotics are probiotics. They play an important role as growth promoters and pathogen inhibitors in poultry industry (Zhang & Kim, 2014). In our study, the highest food conversion rate was recorded in groups 3 and 4. Ashayerizadeh *et al.* (2016) indicated that all levels of probiotic supplementation, except 0.05%, significantly improved ($P<0.05$) feed conversion ratio when compared to control group. Also, Jin *et al.* (1997), Pedron *et al*. (1997), Nezami *et al*. (2000) and Gonzalez *et al*. (2001) reported the

best FCR ratio in chicken raised on high levels of probiotics (5 to 10 g/kg feed). The improved FCR might be due to the maintaining normal intestinal microflora by competitive binding and antagonism, shifting metabolism by rising digestive enzyme activities and by promoting digestion rate of energy nutrients.

The highest geometric mean titre (GMT) of antibodies developed in broiler chicks against *Pasteurella multocida* was recorded in groups 3 and 4. According to Noverr & Huffnagle (2004) and Mohiti *et al.*, (2007) the microbiota plays a vital role in forming the immune system scope. Also Zulkifli *et al.* (2000) reported that birds treated with a *Lactobacillus* culture displayed a higher serum antibody response than oxytetracycline-treated and the control birds. Hamid *et al.* (2006) proved that *Lactobacillus acidophilus* and *Bifidobacterium bifidum*, along with *Streptococcus faecalis* showed considerable increase in growth and immunity in the chicken. On the other hand, Sarwar *et al.* (2019) stated that although the probiotic positive vaccine group showed relatively increased GMT value, no significant differences ($P \leq 0.05$) were found out.

The lowest morbidity and mortality rates after *P. multocida* challenge were recorded in broilers fed feed containing *L. acidophilus, B. subtilis* and *S. cerevisiae* (Group 3) and in those fed on probiotic formulation composed of *L. acidophilus* and *S. cerevisiae* (Group 4). Ramirez *et al.* (2005) and Siwicki *et al.* (2005) stated that the addition of probiotics in rations of chickens led to reduction of the mortality rate. Our data agree with those of Naseem *et al.* (2012) who indicated that the chicken groups supplemented with probiotic showed no mortality and morbidity, but the groups treated with cyclophosphamide or cyclophosphamide plus probiotic presented some sick chicks. Yörük *et al*. (2004) reported that probiotic supplementation (containing *Lactobacillus, Streptococcus, Bifidobacterium* and *Enterococcus* species) during the late laying period in layer hens reduced mortality.

Significant increase in the duodenal and ileal villus height and average crypt depth were recorded in probiotic treated chicks compared to the negative controls. Caspary (1992) stated that the development of intestinal morphology could indicate the health status of the gastrointestinal tract and the increase of the villus height caused increased mucosal surface area and therefore, greater absorption of available nutrients. Ruttanavut & Yamauchi (2010) reported that longer villi in the ileum of adult male layers were associated with slight increase in feed efficiency after dietary supplementation of *Bacillus subtilis* var. natto and in broilers after adding *Enteroccocus faecium* or *Eubacterium* sp. in their feed. Also Abdel-Raheem *et al.* (2012) indicated that the probiotics alter the mucosal architecture in relations of longer villi and enhanced performance in birds. Agboola *et al.* (2015) reported significant ($P<0.05$) increase in the height of villi and crypt depth of birds fed a diet including organic acids or probiotics compared with a non-supplemented diet.

CONCLUSIONS

The present study showed that probiotic formulations, particularly those containing *Lactobacillus acidophilus, Bacillus subtilis* and *Saccharomyces cervisiae* exerted a significant improvement of growth performance (weight gain, feed intake, feed conversion ratio) of broiler chicks. Also the immune response to *Pasteurella multocida* vaccine and the overall morbidity and mortality rates following challenge

with *Pasteurella multocida* in probiotics-fed broilers was significantly improved. The probiotics induced significant morphometric changes in the villous height and crypt depth of the chicken gut that positively influenced absorption of nutrients and exerted positive effect of different growth parameters. The promising and encouraging results of this study highlight the importance of the further evaluation of the management level of the investigated supplements with regard to their positive effects on the gut tissue and therefore, the overall health of broiler chickens.

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