Journal of Advanced Veterinary Research Volume 8, Issue 3 (2018) 49-59

Journal of Advanced Veterinary Research

http://advetresearch.com/index.php/avr/index

AGRIMOS® Prebiotics: Effect on Behavior, Performance, Cecal Microbial Population and Humeral Immunity in Broiler Chickens

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ARTICLE INFO

Original Research

Received:
23 June 2018

Accepted:
14 July 2018

Keywords:

Prebiotics, growth performance, immunity, behavioral tests, heat stress

ABSTRACT

The detrimental impact of summer temperature is a subject of huge concern for poultry health and production in Egypt. This experiment was conducted to evaluate the effects of dietary supplementation of AGRIMOS® prebiotic on performance, internal organs weight, caecal bacterial count and humoral immunity for Avian influenza vaccine and behavioral tests in broiler chickens reared under cyclic heat stress. At day 28; the birds were exposed to 32°C for 9 hours daily after they were randomly allotted to four treatments: 0, 0.5, 2, and 4 g AGRIMOS kg^{-1}, respectively. The experiment showed that, at 42 days of age, 4 g AGRIMOS kg^{-1} significantly improved the birds' performance and significantly increased the abdominal fat, bursa and thymus relative weight as well as, the time of latency to lie and decreased the tonic immobility response. The total aerobic count in the caecal samples showed a significant decrease in all groups received AGRIMOS. However; the lactobacilli count and the Hemagglutination inhibition titers, for avian influenza vaccine, were significantly increased. In conclusion, the AGRIMOS supplement to broiler chickens could be considered a protective prebiotic which control the negative effects of hot environment in summer.

Introduction

Egypt is a subtropical country where the summer climate is considered a major limiting factor for broiler production. Broiler chickens are characterized by rapid growth, short production cycle (35-42 days) and poor heat tolerance. Chickens exposed to ambient temperature above 30˚C from 4 weeks of age up to marketing, suffered from heat stress (Abd-Elsamee, 2005; Ghazalah *et al.* 2008). Heat stress conditions cause decrease in the birds feed intake and feed conversion ratio (FCR) resulting in reduced body weight (BW) gain (Cooper and Washburn, 1998; Yalcin *et al.*, 2003; Ramnath *et al.*, 2008). Liver, heart and gizzard weight were increased while, abdominal fat weight was decreases as a resulted to heat stress exposure (Yahav *et al.*, 1997, Abd El-Gawad *et al.*, 2008). Heat stress suppressed the immunity causing high mortality of birds leading to economic loss in poultry farms (Howlinder and Rose, 1989; Niu *et al.*, 2009). Heat stress could be reflected on the overall animal health and welfare. Measurements of conventional welfare indicators at slaughter age revealed that broilers exposed to stress scored worse on the latency-to-lie test (a test for leg health), and the tonic immobility test (a test for fearfulness) (Van Nuffel *et al.*, 2005; Van Poucke *et al.*, 2007; Mahmoud *et al.*, 2015b). Moreover, heat stress stimulates the proliferation of harmful pathogens including Escherichia, Salmonella, and total aerobic bacteria (Lan *et al.*, 2004; Park *et al.*, 2013).

In healthy chicks the composition of the intestinal microflora remains well established and diet is one of the essential factors that can affect the intestinal microbial ecology (Rehman *et al.*, 2007). Marked changes in the intestinal bacterial composition due to Heat stress has been recorded by Suzuki *et al.* (1983). Modifications of the diets is one of the most preferred and practicable ways to alleviate the negative effects of high environmental temperature in poultry (Mahmoud *et al.*, 2015a). Improving bird health through prebiotic supplementation, a non-digestible food ingredient, selectively stimulates the growth and/or activity of intestinal bacterial population (Gibson *et al.*, 2004). MOS and β-glucan prebiotic has recently received much attention as a dietary supplementation for promoting health in various animals including cattle

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ISSN: 2090-6277/2090-6269/

(Boyd *et al*., 2016), pigs (Nochta *et al*., 2009) and chickens (Awaad *et al*., 2011; Koksal *et al*., 2013). In chickens, MOS and β-glucan prebiotic has been used as immune-stimulant and antioxidant to prevent ochratoxicosis and treating chicken immune dysfunction (Awaad *et al*., 2011), also it is used for reducing the negative effects of delayed feed access on growth rate in broiler chickens (Koksal *et al*., 2013). Supplementation of prebiotic in birds subjected to heat stress is a recent approach to re-establish the normal balance of the intestinal microbial composition (Gibson *et al*., 2004). Prebiotic produce specific changes in the ecology and fermentation profiles of the gastrointestinal microflora (Rehman *et al*., 2008 a,b). Mannan-oligosaccharide (MOS) supplementation has been found to improve the growth of cecal Lactobacillus species in broilers (Baurhoo *et al*., 2007). Some studies about the effect of dietary MOS prebiotic on intestinal microflora, and immune response of broilers under optimal condition have been published recently (Corrigan *et al*., 2011; Kim *et al*., 2011). Supplementations of β-glucan into diet selectively supported the growth of Lactobacilli and Bifidobacteria as well as protected the viability and stabilized the cells of lactobacillus sp. in humans (Battilana *et al*., 2001; Saarela *et al*., 2006).

Among dietary fibers, β-glucans, together with other non-digestible food ingredients such as Mannan -oligosaccharides, are currently being produced commercially for their potential prebiotic effects. This study hypothesized that MOS and β-glucan prebiotic supplementation could reduce the harmful effects of heat stress on performance, ceacal microbial flora and immune system.

Materials and methods

All procedures and protocols were approved by the Faculty of Veterinary Medicine Assiut University, Egypt.

AGRIMOS (Mannan-Oligosaccharides and β-Glucans Combination)

Commercial product AGRIMOS® was purchased from LALLEMAND SAS Co. (19 Rue des Briquetiers, 31702 Blagnac Cedex, France), distributed by Egavet Co., Egypt.

Birds and Husbandry

One hundred sixty eight 1-day-old unsexed chicks of the Ross 308 strain were obtained from a local hatchery (Future poultry, Makram Ebeid St., Nasr City, Cairo, Egypt). The birds were randomly assigned to 12 floor pens (1× 1 m per pen) in the same room at the Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Assiut University during the period from April to May 2016. Wood shavings (5-cm depth) were used as litter. The brooding temperature was 34°C for the first 3 days then gradually reduced by 3°C/wk up to 28 days of age, thereafter, all the chickens were exposed to 32°C for 9 hrs (08:00 – 17:00) daily up to 42 days. Actual pen temperatures and humidity were measured every 4 hrs by using wall mount thermohugrometer, which was fixed 30 cm above the litter surface. All chicks were fed diets formulated according to the requirements proposed by the NRC (1994). A starter diet with 23.43% Crude protein (CP) and 3,050 kcal ME/ kg from day 1 to 14, grower diet with 22.81% CP and 3,150 kcal ME/kg from days 15 to 28, then finisher diet with19.17% CP and 3,200 kcal ME/kg from d 29 to 42. The light regime was 23L: 1D. The birds had free access to feed and water during experimental period.

Experimental Design

At 28 days of age, birds were weighted individually and assigned to 12 floor pens as that each pen average B.W. and weight distribution was not different. The experiment was carried out in a completely randomized design with 4 dietary treatments. In each treatment, there were 3 replicates of 14 birds for each. The experimental groups were as follows; Treatment 1 (control) was fed with a basal diet only, and treatments 2 to 4 were fed with a basal diet supplemented with AGRIMOS® 0.5, 2 and 4 g kg^{-1}, respectively.

Table 1. The ration formulation (Mahmoud *et al*., 2015a)

Ingredient, %	Starter	Grower	Finisher
Corn	52.0	52.3	62.8
Soybean meal,48 % CP	40.0	39.1	29.7
Soy oil	3.59	4.97	4.11
Sodium chloride	0.51	0.46	0.43
DL Methionine	0.30	0.24	0.23
L-Lysine HCL	0.13	- - -	0.07
Threonine	0.06	- - -	- - -
Limestone	1.29	1.15	1.12
Monocalcium phos	1.75	1.48	1.17
Vitamin/mineral premix[1]	0.35	0.35	0.35
Calculated analyses			
Crude protein %	23.4	22.8	19.2
Poultry ME kcal/kg	3050	3151	3200
Calcium %	0.95	0.85	0.75
Available phosphorus %	0.50	0.44	0.36
Methionine %	0.66	0.59	0.53
Methionine + Cystine %	1.04	0.97	0.86
Lysine %	1.42	1.29	1.09
Threonine %	0.97	0.89	0.74
Na %	0.22	0.20	0.19

[1]Provided per kilogram of diet: vitamin A, 13,233 IU; vitamin D3, 6,636 IU; vitamin E, 44.1 IU; vitamin K, 4.5 mg; thiamine, 2.21 mg; riboflavin, 6.6 mg; pantothenic acid, 24.3 mg; niacin, 88.2 mg; pyridoxine, 3.31 mg; folic acid, 1.10 mg; biotin, 0.33 mg; vitamin B12, 24.8 µg; choline, 669.8 mg; iron from ferrous sulfate, 50.1 mg; copper from copper sulfate, 7.7 mg; manganese from manganese oxide, 125.1 mg; zinc from zinc oxide, 125.1 mg; iodine from ethylene diamine dihydroidide, 2.10 mg; selenium from sodium selenite, 0.30 mg.

Data Collection and Sampling

Performance indicators

At 35 and 42 days of age, FI, BW, and BWG were recorded on a pen basis. In addition, FCR, PER, EER, PEF, and BI were calculated.

Protein efficiency ratio (PER): (Kamran *et al*., 2008).
Weight gain divided by protein intake
Energy efficiency ratio (EER): (Kamran *et al*., 2008).
EER= Weight gain×100 / total ME intake
Production efficiency factors (PEF): (Marcu *et al*., 2013)
PEF = Viability (%) × BW (kg) × 100
Age (d) × FCR (kg feed/ kg gain)
Where, BW: body weight; FCR: feed conversion ratio
Broiler Index (BI): (Marcu *et al*., 2013)
BI= Viability (%) × ADG (g/chick/day)
FCR (kg feed/kg gain) × 10

Where, ADG: average daily gain; FCR: feed conversion ratio

Internal organs relative weight

At 42 days of age, 6 birds/ treatment (i.e. 2 birds were ran-

domly taken from each replicate) were euthanized immediately by cutting the jugular vein, and allowed to bleed for approximately 2 min. Viscera were removed immediately; thereafter, heart, liver, gizzard, spleen, bursa of fabricius, thymus and abdominal fat were weighed individually then expressed as a percentage of life B.W.

Caecal microbial examination:

Caecal microbial counting

Ceacum from selected birds were aseptically kept in sterilized plastic bags and freezed at -20°C until analysis. The caecal contents (1 g) of each sample was thawed under room temperature for 15 minutes and examined for enumeration of total bacterial and total lactobacilli count. Caecal contents were serially diluted (10-fold) with physiological saline in 96 wells plate. Ten µL of each dilution were platted on Standard Plate Count Agar (Lab M Limited, Lancashire, UK) and MRS agar plates (Lab M Limited, Lancashire, UK) for total bacterial count and lactobacilli count, respectively. Incubation for total bacterial count and lactobacilli count, was done aerobically at 37°C for 24 hrs and anaerobically at 37°C for 48 hrs (Gas–Pak anaerobic system, Becton Dickinson Microbiology Systems), respectively (Asperger and Saad, 1999). After incubation, colonies were counted and recorded as colony forming units per gram of sample and expressed as log10 cfu g^{-1} (Sieuwerts *et al*., 2008).

Caecal *lactobacillus* spp. identification

Caecal content (0.2-0.4 g) was aseptically collected, transferred to 5 ml Man Rogosa and Sharpe (MRS) broth, and incubated at 37°C anaerobically for 18-24 hrs. After that a loop was streaked on MRS agar plate medium and the inoculated plates were incubated anaerobically at 37°C for 48 hrs. After incubation, each different well-formed colony was randomly picked and streaked onto MRS slope for further identifications. The isolates were subjected for initial identification and morphological examination; Gram staining, oxidase and catalase tests were performed. Only Gram positive bacteria, oxidase and catalase negative were selected to use for the VITEK 2 compact identification after purifying the isolates by sub-culturing them on MRS agar. Suspension preparations were done using a sterile swab or applicator stick and suspend the microorganism in 3.0 mL of sterile saline in clear plastic (polystyrene) test tube. The turbidity was adjusted between 2.7 and 3.3 and measured using the DensiCHEKTM then the ID cards and the tubes were placed into the cassette which was transferred to the VITEK for filling and loading the cards to be able to get the final result report (VITEK®2, 2011).

Hemagglutination inhibition (HI) assay

Each bird received the oil-emulsified inactivated A/chicken/Mexico/232/94 (H5N2) vaccine (vaccine virus titer: 108.5 EID50 or 256 HAU/dose) at the 7th day of age. Blood samples were collected from 24 birds, 6 from each group at the 42nd day of age. Serum samples were stored at −20°C until used. Sample collection and processing were done using proper personal protective equipment (PPE). HI assay was performed as previously described (World Health Organization, 2002). Briefly, serum samples were first inactivated for 30 min at 56°C. Serial two-fold dilutions of serum samples were then incubated with four units of homologous A/chicken/Mexico /232/94 (H5N2) antigen at 37°C for 1 hr. Twenty five microliters of 1% chicken red blood cells (CRBC) were then added and incubated at room temperature for 45 min. The HI titer was defined as the reciprocal of the highest dilution of serum which completely prevented the agglutination of CRBC.

Behavior tests

Latency-to-lie (LTL)

The LTL tests were done using a procedure similar to that of Berg and Sanotra (2003) and Caplen *et al.* (2014). For these tests, 15 birds were randomly taken from each group (5birds/pen) and placed in tubs containing 3 cm of lukewarm water. A stopwatch was used to record the time each bird spent standing before making the first attempt to sit down, at which time the bird was removed and replaced with another bird. A test was terminated if the bird was still standing after 600 s. Two tubs were monitored at 1 time by a single observer. The tubs were surrounded by cardboard walls high enough to prevent test birds from seeing other broilers. LTL testing was carried out by the same person.

Tonic immobility

The birds used for TI measurements were not allowed to have a visual contact with other birds. TI was induced as soon as the birds were caught by gently restraining them on their right side by the legs and wings for 15 s. The experimenter then retreated approximately 1 min and remained within sight of the bird but made no unnecessary noise or movement. Direct eye contact between the observer and the bird was avoided as it may prolong TI duration. A stopwatch was started to record latencies until the bird righted itself. If the bird righted in less than 10 s, it was captured again and the restraining procedure was repeated. If TI was not induced after three trials the duration of TI was considered as 0 s. The maximum duration of TI allowed was 600 s. All TI tests were conducted by the same experimenter (Zulkifli *et al*., 1999).

Statistical Analysis

For performance indicators cage was considered as the experimental unit, while for internal organs relative weight the individual bird was considered as the experimental unit. The data was analyzed by one way analysis of variance using the general linear model procedures of SPSS 16.00 Software (SPSS Inc., Chicago, IL, USA); significance was designated as $P \leq 0.05$. Means were compared by Tukey's test when a significant difference was detected.

Results

During the experimental period, the actual average temperature and relative humidity were 30.5±1.5°C and 40±6%.

The effects of AGRIMOS prebiotic on the performance of broiler chickens exposed to heat stress were shown in Tables 2 and 3. The results clarified that dietary supplemental AGRIMOS prebiotic had no effects on performance at 35 days of age. On the other hand, at 42 days of age the supplementation of diet with 4 g/kg^{-1} AGRIMOS prebiotic significantly ($P \leq 0.05$) increased BW, BWG, PER, EER, PEF, and BI, also it significantly ($P \leq 0.05$) improved FCR, while it did not affect the FI, in compare to control. In contrast, the supplementation of diet with 0.5 or 2 g/kg^{-1} AGRIMOS prebiotic did not affect performance indicators compared to control during the whole experimental period.

The effects of AGRIMOS prebiotic on the internal organs relative weight of broiler chickens exposed to heat stress were presented in Table 4, the results clarified that dietary supple

mental AGRIMOS prebiotic did not affect the relative

Table 2. The effect of different levels of AGRIMOS prebiotic on body weight, body weight gain, feed intake and feed conversion ratio in heat stressed broiler chickens.

Treatments	Body weight (kg)			Body weight gain (kg)			Feed intake (kg feed/bird)			Feed conversion ratio		
	28 days	35 days	42 days	28-35 days	35-42 days	28-42 days	28-35 days	35-42 days	28-42 days	28-35 days	35-42 days	28-42 days
Control	1.18	1.70	2.17^{b}	0.52	0.47	0.99^{b}	1.00	1.00	2.01	1.93	2.19	2.04^{a}
0.5 g/kg diet	1.18	1.73	2.29^{ab}	0.55	0.56	1.11^{ab}	1.03	0.98	2.01	1.96	1.86	1.81^{ab}
2 g/kg diet	1.18	1.73	2.28^{ab}	0.55	0.55	1.10^{ab}	0.98	0.92	1.90	1.76	1.71	1.73^{ab}
4 g/kg diet	1.18	1.90	2.37^{a}	0.72	0.47	1.19^{a}	0.98	0.92	1.90	1.40	2.11	1.60^{b}
SEM	0.017	0.061	0.033	0.061	0.070	0.040	0.019	0.026	0.039	0.186	0.322	0.094
P Value	1.000	0.160	0.021	0.158	0.722	0.049	0.225	0.137	0.129	0.202	0.706	0.058

^{a,b}Means with different superscripts in the same column differ significantly (P ≤0.05).

Table 3. The effect of different levels of AGRIMOS prebiotic on Energy efficiency ratio, Protein efficiency ratio, Production efficiency and Broiler Index in heat stressed broiler chickens.

Treatments	Energy efficiency ratio (EER)			Protein efficiency ratio (PER)			Production efficiency factors (PEF)			Broiler Index (BI)		
	28 days	35 days	28-42 days	28-35 days	35-42 days	28-42 days	28-35 days	35-42 days	28-42 days	28-35 days	35-42 days	28-42 days
Control	16.20	14.92	15.53^{b}	2.73	2.51	2.62^{b}	218.23	231.82	258.21^{b}	386.02	333.21	355.94^{b}
0.5 g/kg diet	16.74	17.91	17.30^{ab}	2.82	3.02	2.91^{ab}	225.66	278.84	302.03^{ab}	441.42	484.43	439.56^{ab}
2 g/kg diet	17.76	18.45	18.11^{ab}	2.99	3.11	3.05^{ab}	239.23	291.78	314.63^{ab}	450.87	465.14	456.23^{ab}
4 g/kg diet	23.13	15.83	19.57^{a}	3.89	2.67	3.30^{a}	311.65	271.53	353.72^{a}	788.95	356.42	534.07^{a}
SEM	1.92	2.44	0.76	0.32	0.41	0.13	26.16	31.63	16.85	113.04	112.02	32.78
P Value	0.112	0.711	0.033	0.112	0.711	0.033	0.117	0.595	0.025	0.121	0.717	0.031

^{a,b}Means with different superscripts in the same column differ significantly (P ≤0.05).

weight of heart, liver, spleen and gizzard. On the other hand, abdominal fat, bursa of fabricius and thymus percentages were increased (P< 0.001) in all AGRIMOS prebiotic treated groups in compare to the control.

Effect of AGRIMOS on caecal bacterial content:

The effect of AGRIMOS supplementation on the total mesophilic count of broiler chickens exposed to cyclic heat stress are presented in Fig. 1(A). A highly significant difference (p< 0.0001) was observed between control and other treatment groups. The count in samples collected at day 42 showed a significant decrease (P<0.0001) in all groups given different AGRIMOS concentrations in compare with the control group. Additionally, the lowest count (4.95 log10/g) was observed in the group with the highest AGRIMOS concentration (4 g /kg feed).

Data presented in Fig.1 (B) showed the effect of AGRIMOS supplementation on the lactobacilli count of the collected caecal contents. A highly significant difference (p=0.006) in the count was observed between the groups received AGRIMOS supplementation and the control group. There was an increase in the count in groups with AGRIMOS supplementation than the control. Group received 4g AGRIMOS/kg in diet showed the highest lactobacilli count (highly significant, P=0.008) and the count was 9.6 log10/g. VITEK ®2 compact system provides highly accurate and reproducible results for phenotypic automated microbial identification (Pincus, 2010). Results for lactobacillus identification indicated the presence of Lactobacillus parabuchneri in both the control and first treatment which received AGRIMOS® 0.5gkg^{-1} diet. However, *Lactobacillus acidophilus* and *Lactobacillus gasseri* were found in the ceacal samples of the second and the third treatment groups which received AGRIMOS® 2 and 4 gkg^{-1} diet, respectively.

Effect of AGRIMOS on HI titer

The effect of AGRIMOS prebiotic supplementation on the HI titer of Avian influenza vaccine at the end of experiment from broiler chickens exposed heat stress were presented in Fig. 2. There was a highly significant difference between treatment groups and control group. The average HI titer was 5.5 log^2 in control (Basal diet), 6.7log^2 in Group 1; Basal diet + 0.5 g AGRIMOS /kg diet, 7.3 log^2 in Group 2; Basal diet + 2g AGRIMOS /kg diet, and 8 log^2 in Group 3; Basal diet + 4g AGRIMOS /kg diet.

Effect of AGRIMOS on Latency-to-Lie Test and tonic immobility

The effects of AGRIMOS prebiotic on the time of Latency-to-Lie and tonic immobility Tests in broiler chickens exposed to heat stress are shown in Table 5; the results clarified that at 42 days of age the supplementation of diet with 4 g/kg^{-1} AGRIMOS prebiotic significantly (P≤0.05) increased the time of

Table 4. The effect of different levels of AGRIMOS prebiotic on internal organs relative weight in heat stressed broiler chickens.

Treatments	Liver (%)	Heart (%)	Gizzard (%)	Spleen (%)	Abdominal fat (%)	Bursa of Fabricius (%)	Thymus (%)
Control	2.02	0.47	1.78	0.12	1.05^{b}	0.11^{c}	0.25^{c}
0.5 g/kg diet	2.22	0.53	1.67	0.13	1.58^{a}	0.16^{ab}	0.29^{bc}
2 g/kg diet	2.13	0.49	1.65	0.10	1.47^{a}	0.18^{a}	0.34^{ab}
4 g/kg diet	2.14	0.44	1.52	0.12	1.61^{a}	0.14^{b}	0.35^{a}
SEM	0.09	0.03	0.07	0.01	0.07	0.01	0.02
P Value	0.495	0.252	0.082	0.120	0.000	0.000	0.001

^{a,b}Means with different superscripts in the same column differ significantly (P ≤0.05).

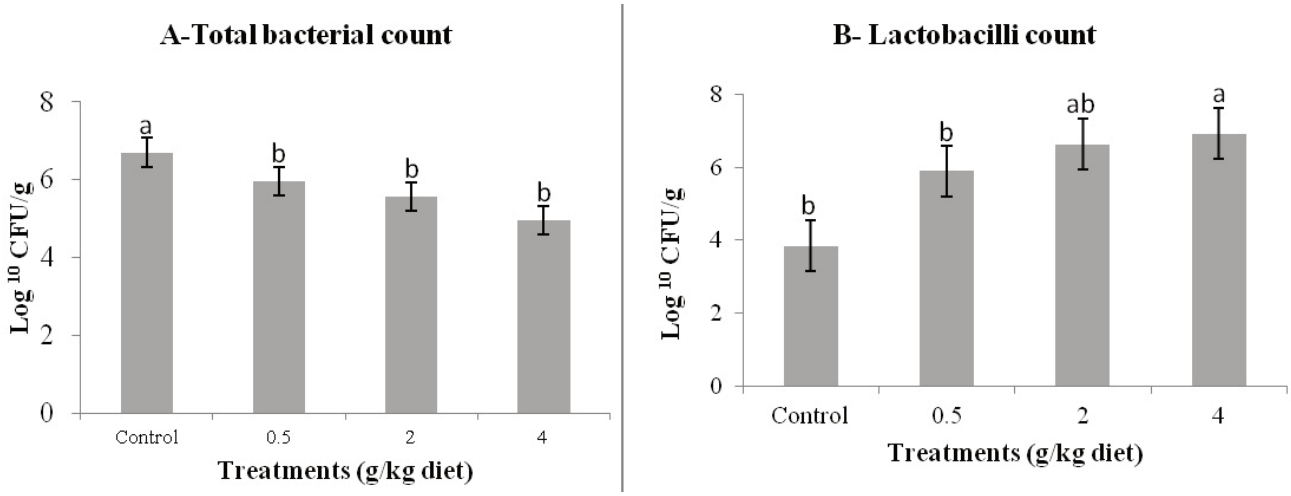

Fig. 1 (A and B). Log10 CFU of total aerobic and lactobacilli counts in caecal content of broiler chicken collected at the end of experiment as shown in a and b, respectively. The groups were as follow: Control (Basal diet), Group 1; Basal diet + 0.5 g AGRIMOS /kg diet, Group 2; Basal diet + 2g AGRIMOS /kg diet, Group 3; Basal diet + 4g AGRIMOS /kg diet; abc: means with different letters are significantly different (p<0.05)

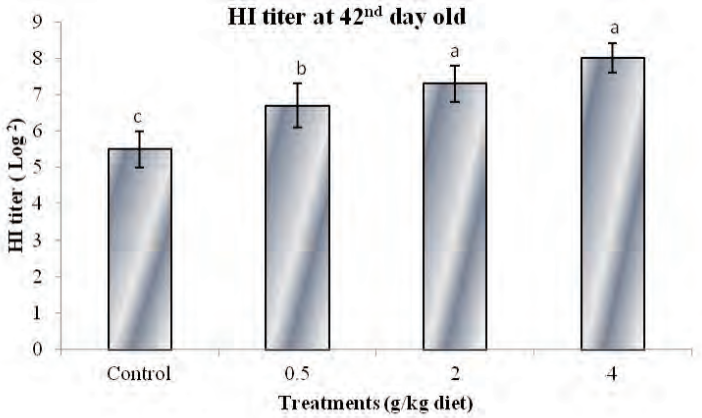

Fig. 2. The effect of AGRIMOS prebiotic dietary supplementation on the HI titer of Avian influenza vaccine. The average HI titer was 5.5 log^2 in control (Basal diet),, 6.7log^2 in Group 1; Basal diet + 0.5 g AGRIMOS /kg diet, 7.3 log^2 in Group 2; Basal diet + 2g AGRIMOS /kg diet, and 8 log^2 in Group 3; Basal diet + 4g AGRIMOS /kg diet.

Latency-to-Lie test in compare to the control. On the other hand, the time of Tonic immobility Test was decreased ($P < 0.001$) in all AGRIMOS prebiotic treated groups in compare to the control.

Table 5. Effect of AGRIMOS Combination on Latency-to-Lie Test and tonic immobility in Broiler Chickens Exposed to Heat Stress.

Treatments	Latency-to-Lie Test (sec)	Tonic Immobility (sec)
Control	74.00^{b}	143.33^{a}
0.5 g/kg diet	147.00^{ab}	57.83^{b}
2 g/kg diet	146.33^{ab}	23.67^{b}
4 g/kg diet	222.00^{a}	19.00^{b}
SEM	28.015	13.77
P value	0.025	0.001

^{a,c} Means with different superscripts in the same column differ significantly ($P \leq 0.05$).

Discussion

The current experiment findings clarified that dietary supplementation of diet with 4 g/kg^{-1} AGRIMOS prebiotic improved the performance indicators (BW, BWG, PER, EER, PEF, and BI) in broiler chickens exposed to heat stress. These results could be supported by the findings of Sohail, *et al.* (2012), which revealed that dietary supplementation of MOS increased growth performance and decreased corticosterone concentrations in heat stressed broiler chickens. Similarly, Shendare *et al.* (2008) reported that inclusion of 1 g/kg^{-1} AGRIMOS prebiotic in the diet of broiler chickens reared under normal recommended environmental temperature significantly increased the body weight gain and improved the feed efficiency of birds as compared to the control diet. These findings disagree with pervious works reported that MOS and β-glucan prebiotic inclusion had no beneficial effect on growth rate and feed consumption in broiler chickens exposed to post hatch holding time stress (Cengiz *et al.*, 2012; Koksal *et al.*, 2013). Regarding the internal organs relative weight, the current experiment clarified that AGRIMOS prebiotic did not affect the hearts, gizzard, liver, and spleen weight. Similarly, Cengiz *et al.* (2012) pointed out that dietary supplementation of 1g/kg MOS and β-Glucans prebiotic for broiler chicks suffered from delayed feed access stress did not affect the hearts, gizzard, liver, and spleen weight. Also, Yalçin *et al.* (2014) reported that dietary supplementation with 0, 1, 2 and 3 g/kg yeast cell wall (InteMos) not affect the relative weights of gizzard, liver and heart in Ross 308 male broiler chicks reared under the normal recommended temperature. On contrary, Sadeghi *et al.* (2013) reported that inclusion of 1 g/kg MOS and β-Glucans prebiotic to the diet of Salmonella enteritidis challenged chicks increased the relative weight of the spleen. In addition, the current experiment indicated that dietary supplementation of AGRIMOSs prebiotic at 4g/kg increased the abdominal fat, bursa of Fabricius and thymus relative weight. These results were in agreement with (Awaad *et al.*, 2011; Barros, *et al.*, 2015). Where, Awaad *et al.* (2011) recorded that dietary supplementation of 2 g/kg MOS and β-Glucans prebiotic increased the relative weight of bursa in Ochratoxicated Broiler Chickens. Barros, *et al.* (2015) mentioned that dietary supplementation of MOS at 1.5, 1, and 0.5 g/kg for the periods of 1 to 21; 22 to 33, and 34 to 42 days of age, respectively, increased the abdominal fat content in male Cobb broiler chicks raised under normal recommended temperature.

It is already established that hypothalamic pituitary adrenal (HPA) axis activation have been responsible for the reduced performance and low lymphoid organs' relative weights in broiler chickens exposed to heat stress (Quinteiro-Filho *et al.*, 2010). Based on the fact that dietary supplementation of MOS reduce the detrimental effects resulted from HPA axis activation in heat stressed broiler chickens as it decreased the serum cortisol and cholesterol concentrations and increased thyroxine concentration and improved humeral immunity (Sohail, *et al.*, 2010).

Broiler intestinal tract harbors a diverse and dynamic population of microflora, living in symbiotic relationship with the host, which is important for nutrition, metabolism, and immunity (Sohail *et al.*, 2010). Rearing chicks under heat stress conditions has been shown to cause marked changes in the composition of the intestinal microbial community (Burkholder *et al.*, 2008). Exposure to extreme temperature is associated with increased intestinal colonization and fecal shedding of pathogens in poultry (Bailey, 1988). In the current study, the total bacterial count in the collected caecal samples showed a significant decrease in all treatment groups received different concentrations of AGRIMOS. Cereal β-glucan significantly increased the population of *Lactobacillus* and *Bifidobacterium*, whereas the number of *Enterobacteriaceae* decreased in a dose-dependent manner during the administration period (Shen *et al.*, 2012).

Pourabedin *et al.* (2014) revealed that MOS supplementation did not affect the total bacteria population in broilers raised under suboptimal growing conditions (high stocking

density and mild cold temperature). Oligosaccharides and related carbohydrates are not subjected to degradation or hydrolyzes in the upper intestinal tract (Hidaka *et al*., 1986; Oku, 1986). Therefore, the inhibition and modulation of the bacterial community in the chick intestinal tract can be explained, by the microbial fermentation of these oligosaccharides in the caecum and colon. In addition, the ability of microbe to attach to the host cell surface is essential for it to colonize and cause enteric disease and that adhesion can be mediated by fimbriae on the bacterial cell surface. Gram-negative bacteria with mannose-specific (Type 1) fimbriae are able to attach to and then colonize the intestinal wall. MOS is an oligosaccharide with a terminal mannose moiety and these mannose units mimic the receptors found on enterocytes on which the Gram-negative bacteria are able to bind. Therefore, MOS and other oligosaccharides can serve as trap attachment sites for Gram-negative bacteria, thereby preventing attachment onto enterocytes and subsequent enteric infection. Newman (1994) reported that the presence of dietary MOS in the intestinal tract removed bacteria that could attach to the lumen of the intestine, which might provide a more favorable environment for nutrient utilization by the bird (Savage and Zakrzewska, 1996). Dietary MOS supplementation has been reported to improve the broilers' condition of stress due to high stocking density (Hooge *et al*., 2003). In this respect, the dietary supplementation of AGRIMOS can diminish some of the detrimental effects of high environmental temperature in broilers through modulation of gut microflora.

Lactobacilli, is thought to create conditions unfavorable to the growth of pathogens (Tamura, 1983). Lactobacilli can compete with pathogens, release bacteriocidal or bacteriostatic chemicals, maintain intestinal immune homeostasis and prevent inflammation (Cebra, 1999 and Lan *et al*., 2005). Stress can disturb the balance of the intestinal microflora leading to decreased lactobacilli proportion (Selig and Patterson, 2004; Lutgendorff et al., 2008). The result obtained from the current study revealed that broilers exposed to high environmental temperature and received AGRIMOS showed a significant increase of Lactobacilli population in the caeca. High environmental temperatures alter the activity of the neuroendocrine system of poultry, resulting in activation of the hypothalamic–pituitary–adrenal (HPA) axis, and elevated plasma corticosterone levels (Quinteiro–Filho et al., 2010; Quinteiro–Filho *et al*., 2012). It was observed that stressed chicks due to high temperature exhibit elevated plasma levels of corticosterone (Lin *et al*., 2006), that harms growth and impairs the immune system (Sohail *et al*., 2010; Haldar *et al*., 2011). A link between HPA axis, the intestinal microflora and stress has been described (Gareau *et al*., 2007; Rhee *et al*., 2009). It is hypothesized that the intestinal microflora may stimulate sensory neurons and production of cytokines (Kawahito *et al*., 1994). These cytokines and certain unknown factors secreted from entero-chromaffin cells may influence adrenal gland function by lowering circulating corticosterone levels. The beneficial effects of probiotic Lactobacillus in broilers reared under heat stress have been reported (Lan *et al*., 2004; Rahimi and Khaksefidi, 2006). In addition, increased Lactobacilli population in the cecal contents of broiler supplemented with MOS and reared under optimum condition was investigated (Baurhoo *et al*., 2009; kim *et al*., 2011). However, to our best knowledge, no literature is available concerning the prospective effects of prebiotic AGRIMOS on the composition of the intestinal microbial community and HPA axis activity in broilers reared under high environmental temperature. Therefore, it can be hypothesized that feeding of a prebiotic AGRIMOS may be helpful in improving the adverse effect of high temperature in broilers (HPA axis and blood corticosterone level) through restoring the intestinal microbial ecology.

During the same experiment, broilers showed a significant low blood cortisone level compared with the control group (unpublished data). According to Ahn *et al*. (2003), serum cholesterol level has been reduced due to bile salt hydrolase activity of *Lactobacilli*. Similar results was observed in rat by Oyetayo *et al*. (2003) who found that *Lactobacilli* have direct effect on cholesterol levels by assimilation and elimination from the growth media. It can be concluded that lactobacillus has the ability to lessen the harmful effect of activated HPA axis and subsequent cortisone level in broilers subjected to cyclic heat stress. Finally, MOS and β- Glucan, have been shown to stimulate beneficial bacteria while also having a negative effect on the total bacterial load in the broiler gut, which can have a positive effect on the birds' health. Therefore, commercial AGRIMOS supplement may provide advantages by stabilizing the gut microflora that acted directly or indirectly by producing some factors that are absorbed from the gut and altered HPA axes.

Lactobacillus acidophilus and *Lactobacillus gasseri* identified in the ceacal samples of 42 day old broilers exposed to cyclic heat stress. Wang *et al*. (2014) could isolate *L. acidophilus* and *L. gasseri* out of 12 *Lactobacillus* spp. from the intestinal microflora of chickens by 16S ribosomal DNA (rDNA) targeted probes, and proposed that *L. acidophilus* was one of the typical and predominant *Lactobacillus* species present in the gut (Wang *et al*. 2014). Also, Bjerrum *et al*. (2006) could isolate *Lactobacillus gasseri* from the microbial community of the caecum in conventional and organic broiler chickens (Bjerrum *et al*., 2006).

L. acidophilus has a wide range of health benefits as it has the ability to lowers total blood cholesterol, LDL-cholesterol, and total liver cholesterol and liver TAG (Huang *et al*., 2010) and promotes gastric ulcer healing in rats (Khoder *et al*., 2016), In mice studies, *L. acidophilus* boosts immunity by enhancing natural and acquired immunity (Gill *et al*., 2000) as well as it combats oxidative stress and molecular alterations associated with aging (Kaushal and Kansal, 2012), ameliorate GI infections as it alleviates *E. coli* infection (Kumar *et al*., 2016), inhibits the growth of *C. difficile, Salmonella enterica* (Yun *et al*., 2014) and protects against influenza virus (H1N1) infection (Goto *et al*., 2013). Bacteria-induced colitis can effectively been prevented by *L. acidophilus* through limiting infection and promoting mucosal protective regulatory immune responses (Chen *et al*., 2005), as well as, suppressed all of the 74 gram-negative and 16 of gram-positive bacteria found in burn wounds in mice (Jebur, 2010).

Meanwhile, *L. gasseri* involves in lots of health profits including increased energy expenditure, reduced blood glucose, improved glucose tolerance and attenuated inflammation in rats (Shirouchi *et al*., 2016). Enhances immunity in the elderly by increasing the number of CD8(+) T cells and reduces CD28 expression loss in CD8(+) T cells (Miyazawa *et al*., 2015) as well as, increases IgA levels in breast milk and reduces the incidence of diarrhea in mouse pups with rotavirus infection (Kadooka *et al*., 2012). *L. gasseri* as a probiotic protected mice against the influenza virus and ameliorated infection symptoms by stimulating local and systemic immune responses (Nakayama *et al*., 2014, Kawase *et al*., 2012). In addition, protection the body from different pathogenic bacteria exposure including, Clostridium in human (Sugawara *et al*., 2016), enteropathogenic *E. coli* (Yoda *et al*., 2014) and both *H. pylori* and *H. suis* infections in mice (Matsui *et al*., 2015).

Dietary supplementation of mannan-oligosaccharide increased local mucosal IgA secretions; humoral and cell-mediated immune responses (Gómez-Verduzco, *et al*, 2009) beta-glucan exposure increased interleukin-1 (IL-1) production as well as induced macrophage to proliferate. Dietary beta-glucan supplementation increased the macrophage phagocytic activity, Furthermore, the primary and secondary lymphoid organs such as bursa of Fabricius, thymus and

spleen were larger in beta-glucan-supplemented chicks as compared to the chicks on basal diet (Guo, etal 2003). The findings of our study showed that AGRIMOS improves the immune responses in the chicken.

High environmental temperature is an important predisposing factor to leg weakness and deformities (Hester, 1994; Oviedo-Rondón, *et al*., 2009) due to its effect on mineral and vitamins absorption and metabolism. Latency to lie (LTL) is a behavioral tool used to assess the leg weakness in broilers (Weimer *et al*., 2016). The current study results clarified that the supplementation of diet with 4 g/kg^{-1} AGRIMOS prebiotic significantly increased the time of latency to lie test at 42 days of age in comparison to the control group. No direct explanation could be found for this increase (no extra leg culls or mortality occurred in this group before testing), but analysis of behavioral data published by Mahmoud *et al*. (2017) showed that the groups of 4 g/kg AGRIMOS had 25% higher walking activities than the control group. This extended exercise may have led to the increased leg strength because training is known to increase bone density and decrease bending and twisting resulting in improving the leg health and strength (Reiter and Bessei, 1998).

Tonic immobility duration is a traditional behavioural measure to stress in poultry (Gallup, 1979). It is already recorded that elevated air temperature increased the duration of tonic immobility response in Ross 308 broilers (Skomorucha *et al*., 2010); indicating higher fearfulness (Altan *et al*., 2003). The current study results clarified that the time of Tonic immobility Test was decreased in all AGRIMOS prebiotic treated groups in compare to the control. Possibly indicating that birds of control group is more sensitive to heat stress compared to prebiotic treated birds.

The current experiment suggested that improving broiler chicken performance, and increased bursa, thymus and abdominal fat weight in birds fed the AGRIMOS may be attributed to inhibition of pathogenic microorganisms' colonization in the gastrointestinal tract and development of healthier intestinal microflora and epithelial cells thereby increasing the absorption and utilization of the dietary nutrients (Yang *et al*., 2008; Youssef *et al*., 2011; Ghasemian and Jahanian, 2016). In addition to, its antioxidant, and potent immunomodulatory effect (Shendare *et al*., 2008; Awaad *et al*., 2011; Barros, *et al*., 2015).

Conclusion

Taking into consideration the heat stress condition under which this experiment was carried out, it is possible to conclude that the inclusion of MOS and β-glucan prebiotic at 4 g/kg diet is recommended. It shows significantly higher performance efficiency and increased lymphoid organs relative weight and exhibited the lowest bacterial count and the highest lactobacilli count. In conclusion, dietary AGRIMOS supplementation can reduce some of the detrimental effects of high temperature in broilers. However, further investigation still required for standard approval of AGRIMOS supplementation as a natural additive, for efficient treatment the deleterious effect of heat stress.

Competing interests

The authors declare that they have no competing interests.

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