Effects of dietary inclusion level of a mixture of probiotic cultures and enzymes on broiler chickens immunity response

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Abstract The current study was conducted to analyse the effect of a mixture of probiotic cultures and enzymes (Probio Enzyme, Xvet GmbH, Germany) on the immune response and weight of central lymphoid organs and liver in broilers. A total of 270 male chickens were randomly divided into nine groups, with three replicates of 10 birds each. Treatment groups were fed for either 22 or 42 days with different levels of Probio Enzyme 250, 500, 750 and 1000 g/ton, whereas the control group fed a basal diet without Probio Enzyme. To analyse the effects of dietary supplementation on broilers humoral immune response, the antibodies titres for avian influenza (AI) and Newcastle disease virus (NDV) and to the sheep red blood cells (SRBC) challenge were assayed in birds from each experimental unit, along with the assessment of the weight of the main lymphoid organs and

liver. The addition of the Probio Enzyme mixture did not significantly affect the titres of the antibodies against AI and NDV at day 42, despite the wide individual variation observed specially on the antibody titres at day 33. Treatments affected the production of IgG after the second challenge with SRBC ($P = 0.003$), which was transposed to the correspondent total Ig titres ($P = 0.044$). Conversely, a lower birds' body weight (BW) was found in the majority of treated groups compared to control ($P = 0.031$). The spleen was the only lymphoid organ showing differences in the absolute and relative weight ($P = 0.003$ and $P = 0.001$, respectively). No differences were found in thymus and Bursa of Fabricius weights. In conclusion, broilers treated with Probio Enzyme showed a satisfactory immune response compared with control, despite the wide variation found after the first vaccine challenge against AI. Moreover, the probiotic mixture dose and duration modulated differently the immune response and the spleen weight, unaffected the central lymphoid organs weight.

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Introduction

Probiotic supplement for the development and stabilization of the intestinal flora is used particularly before birds' exposure to expected stressors such as transportation, new housing allocation or feed changes (Gaggia et al. 2010; Jahromi et al. 2016). Although the gut microbiome is quite complex, comprising a multitude of bacteria, yeast and protozoa, various species of lactobacilli play a major role maintaining the equilibrium of intestinal biota and preventing the colonization of pathogens (Bai et al. 2013; Chen et al. 2014) such as pathogenic *Escherichia coli* and *Salmonella* spp. In parallel,

particular mixtures of probiotic cultures increase feed conversion, growth performance and meat quality, increasing therefore the productivity of poultry (Gaggia et al. 2010). This is possibly due to the fact that microorganisms naturally living in the intestinal tract are crucial for poultry digestion and immunity. However, conflict reports on the use of probiotic cultures suggest that influences may exist associated to the composition of the culture, the dose and/or duration of use (Gaggia et al. 2010; Rezaei et al. 2015).

Industrial produced enzymes could aid the breakdown of a wide variety of feed components that typically would be left unused. A combination of lactobacilli and enzymes increases the efficacy of the digestion of different ingredients in feed (Chen et al. 2014); hence, it could improve feed conversion rate, increase the release of essential nutrients and reduce faecal losses and thus lower manure production, as well as less phosphorus and nitrogen excretion (Jin et al. 2000; Yang et al. 2009; Wang and Gu 2010). The effect of gut flora and immunity is presently a well-studied theme in human medicine (Guarner and Malagelada 2003; Macpherson and Harris 2004), where various mixtures with microorganisms, the so called probiotics, are used to improve or stabilize the gut microbiome. Probiotics and exogenous enzymes can also enhance broiler productivity and likely immunity too, that may represent an alternative to the use of sub-therapeutic doses of antibiotics (Talebi et al. 2008), meeting the growing public concerns on the contribution of this practice to the increasing antibiotic resistance. To date, information on the effect of a mixture of probiotic microorganisms and enzymes on the immunity of broilers are missing, to the authors' best knowledge. Therefore, the present study intended to determine the effect of different dietary levels of a probiotic-enzyme supplementation to broiler chickens on their immune competence.

Material and methods

Animals and housing

A total of two hundred seventy (270) 1-day-old Ross 308 (Aviagen, Newbridge, Scotland, UK 35805) male broiler chickens were purchased from a commercial hatchery. The chickens were placed in cages with dimensions of 1.0×1.0 m, providing a floor area of 0.1 m^2 per bird, in a thermostatically controlled curtain sidewall poultry barn. Paper rolls were used for cage embedding. The birds were kept in cages, 10 chickens (41 ± 0.5 g) per cage, throughout of the study period (42 days). Each cage was assigned to a dietary treatment.

Heat from thermostatically controlled gasoline rocket heaters was used to maintain constant the ambient temperature within the poultry barn; temperature at 33°C at placement and decreased periodically to 23°C at 3 weeks of age, remaining thereafter constant until the end of the research period. The

barn atmosphere was humidified via a water spray to preserve relative humidity between 55 and 65%. A 23-W fluorescent tubes in ceiling fixtures provided a constant 24 h light cycle on day one that was changed into a 21 h/day after day two until the end of the experiment. Forced air ventilation within the poultry barn was facilitated by wall-mounted fans on each end of the barn, establishing a tunnel ventilation.

Feed and treatments

A two-phase feeding program was used in this trial, consisting on a provision of starter diet from 1 to 21 days and grower diet from 22 to 42 days (Table 1). The diets met or exceeded Ross 308 catalogue recommendations (Table 2). A commercially available probiotic supplement with concentrated enzymes (Probio Enzyme, XVET GmbH, Germany) was administered to broilers through the feed. The product's composition included cultured *Bacillus licheniformis*, *Bacillus subtilis*, *Enterococcus faecium*, *Lactobacillus acidophilus*, β -glucanase (3.2.1.4), β -glucanase (3.2.1.6), β -xylanase (3.2.1.8), α -amylase (3.2.1.1), protease and cellulose.

The effect of treatments was studied by a 4×2 factorial arrangement with four dietary Probio Enzyme levels (250, 500, 750 and 1000 g/ton) and two durations of treatment (14 and 42 days). Additionally, a control treatment without dietary Probio Enzyme supplementation was used. The trial comprised nine treatments with three replicates for each treatment, whereby each replicate included ten birds, as follows:

Treatment 1: T1, standard diet + 250 g/ton of Probio Enzyme for 14 days

Table 1 Feed ingredients of used diets during the starter (1–21 days of age) and grower (22–42 days of age) periods

Ingredient (g/kg)	Starter period	Grower period
Corn	54.32	58.69
Soybean meal (44% CP)	39.43	31.87
Soybean oil	2.16	5.83
Dicalcium phosphate	2.05	1.68
Sodium chloride	0.37	0.37
DL-methionine	0.20	0.22
Lysine hydrochloride	0.07	0.05
Vitamin premix ^a	0.25	0.25
Mineral premix ^b	0.25	0.25
Mineral oysters	0.90	0.79

^a Supplied per kilogram of diet 12,000 IU vitamin A, 10 mg vitamin E, 2200 IU vitamin D, 35 mg niacin, 12 mg D-pantothenic acid, 3.63 mg riboflavin, 3.5 mg pyridoxine, 2.4 mg thiamine, 1.4 mg folic acid, 0.15 mg biotin and 0.03 mg vitamin B

^b Supplied per kilogram of diet 60 mg manganese, 40 mg zinc, 1280 mg iron, 8 mg copper, 0.3 mg iodine and 0.2 mg selenium

Table 2 Nutrient analysis of the diets during the starter (1–21 days of age) and grower (22–42 days of age) periods

Nutrient analysis	Starter period	Grower period
Metabolizable energy (kcal/kg)	2900	3200
Crude protein (%)	22.16	19.02
Calcium (%)	1.00	0.85
Available phosphorus (%)	0.50	0.42
DCAB (mEq/kg)	236	202
Lysine (SID*) (%)	1.15	0.96
Methionine (SID) (%)	0.50	0.48
Methionine + cysteine (SID) (%)	0.83	0.78
Threonine (SID) (%)	0.79	0.71

* SID standardized ileal digestible

Treatment 2: T2, standard diet + 500 g/ton of Probio Enzyme for 14 days

Treatment 3: T3, standard diet + 750 g/ton of Probio Enzyme for 14 days

Treatment 4: T4, standard diet + 1000 g/ton of Probio Enzyme for 14 days

Treatment 5: T5, standard diet + 250 g/ton of Probio Enzyme for 42 days

Treatment 6: T6, standard diet + 500 g/ton of Probio Enzyme for 42 days

Treatment 7: T7, standard diet + 750 g/ton of Probio Enzyme for 42 days

Treatment 8: T8, standard diet + 1000 g/ton of Probio Enzyme for 42 days

Treatment 9 (control): T9, standard diet without Probio Enzyme for the entire period

Vaccination program

Birds were vaccinated at 1st, 11th and 19th days of age against Newcastle disease virus (NDV) and on the 1st and 19th days of age against infectious bronchitis. Gumboro vaccination was at 14th and 25th days of age. Avian influenza (AI) vaccination was given at 1st day of age.

Immunity assays

The measurement of the magnitude of the immune response was performed as described by Pourhossein et al. (2015). Briefly, at 10 and 28 days of age, sheep red blood cells (SRBC) suspension (5% phosphate-buffered saline) was injected into the breast muscle of six birds per treatment. Fourteen days after each sensitization (24 and 42 days, respectively) antibody titres against SRBC were determined. Total serum antibody titres to SRBC were determined by hemagglutination assay. In U-bottom microtiter plates, two-

fold serial dilutions of heat-inactivated (at 56 °C) serum in PBS (0.01 mol/L, pH 7.4) were made to measure the total antibody or in PBS with 1.4% 2-mercaptoethanol for the determination of immunoglobulin G (IgG) antibody titres. All antibody titres were recorded as ²log of the highest dilution of serum that agglutinated an equal volume of a 0.5% SRBC suspension in PBS. The IgM titre was determined by the difference between total and IgG titre.

From each treatment group, three birds were randomly chosen for blood sampling, collected from the brachial vein. Serum was separated by centrifugation at 1300×g for 15 min following a 1 h incubation at room temperature and stored at −20 °C until the analysis. The antibody titres against NDV and AI were measured by hemagglutination-inhibition test according to Cunningham (1971). Blood sampling was performed on days 17 (for NDV), 33 (for AI) and 42 (for NDV and AI), respectively. At the end of the experiment, at 42 days, three representative birds from each treatment were randomly chosen and killed by cervical dislocation. The final defeathered body weight (BW), as well as the weights of liver, thymus, spleen and bursa of Fabricius were recorded for the estimation of their relative weight.

Statistical analysis

Statistical computations were performed by using a statistical software (SPSS Statistics 1997, version 22.0 for Mac OS-X-10.8). The normal distribution of data for each category of the independent variables was rejected by Kolmogorov-Smirnov and Shapiro-Wilks tests. Hence, the Kruskal-Wallis nonparametric method was used to determine the differences among treatments for the immune parameters tested in the current study. The response to SRBC and immune response against AI and NDV were analysed after the 2nd vaccination for pattern recognition as the use of the medians of the organs' weight per treatment.

Results

The results from this study are summarized in Tables 3, 4 and 5, respectively. In overall, antibody titres for AI and NDV were independent by the treatment duration, except for the AI titres at day 33 ($P = 0.027$) and by the amount of the Probio Enzyme complex used in these experiments (Table 3). Wide individual variations were recorded within the treatment groups for both the influenza and the NDV antibody titres by day 33. Nevertheless, at day 33, most of the birds had produced antibodies against AI, while at day 42, all birds showed an active immune response with titres varying from 1:8 to 1:16. The vaccine-induced antibody titres did not differ among the nine treatment groups for influenza or NDV (Table 3). At day 17, most birds had no antibody titres against

Table 3 Influence of Probio Enzyme treatments on the immune response after vaccination (avian influenza and Newcastle disease virus) of broilers. Values represent the median [mean rank]

Treatments		Antibody titres against AI		Antibody titres against NDV	
		Day 33	Day 42	Day 17	Day 42
Length (day)	Control	2 [15.33] ^{a,b}	3 [11.00]	0 [12.50]	4 [13.50]
	Short (14)	1 [9.83] ^a	4 [15.50]	0 [13.62]	4 [14.42]
	Long (42)	2 [17.83] ^b	3.5 [13.25]	0.5 [14.75]	4 [13.78]
<i>P</i> value		0.027	0.522	0.850	0.966
Probio Enzyme (g/ton)	0	2 [15.33]	3 [11.00]	0 [12.50]	4 [13.50]
	250	2 [17.83]	4 [15.50]	0 [12.50]	4 [17.25]
	500	1 [11.67]	4 [15.50]	0.5 [14.75]	4 [11.50]
	750	1 [9.83]	3.5 [13.25]	0.5 [14.75]	4 [16.75]
	1000	1.5 [16.00]	3.5 [13.25]	0.5 [14.75]	4 [10.75]
<i>P</i> value		0.318	0.861	0.957	0.435
T1		1 [14.17]	4 [15.50]	1 [17.00]	4 [16.17]
T2		0 [5.50]	4 [20.00]	0 [12.50]	4 [12.17]
T3		0 [5.50]	3 [11.00]	1 [17.00]	4 [15.83]
T4		1 [14.17]	4 [15.50]	0 [8.00]	4 [13.50]
T5		2 [21.50]	4 [15.50]	0 [8.00]	4 [18.33]
T6		2 [17.83]	3 [11.00]	1 [17.00]	4 [10.83]
T7		1 [14.17]	4 [15.50]	0 [12.50]	4 [17.67]
T8		2 [17.83]	3 [11.00]	1 [21.50]	3 [8.00]
T9 (control)		2 [15.33]	3 [11.00]	0 [12.50]	4 [13.50]
<i>P</i> value		0.115	0.225	0.238	0.270

Different superscripts within columns differ significantly at $P < 0.05$

NDV, but at day 42, all the birds had titres varying from 1:4 to 1:1024.

Alike the observed on the antibody titres for influenza and NDV, a wide variation was also observed on the response to inoculation of SRBC (Table 4). In general, the ability to produce immunoglobulins against the xenogeneic erythrocytes was not affected neither by the length of treatment nor the dose of the Probio Enzyme mixture. However, statistical differences were found between the groups regarding IgG production after the first and second inoculation ($P = 0.005$ and $P = 0.003$, respectively) and also in the total immunoglobulins produced after the second inoculation ($P = 0.044$). The total Ig produced after the first inoculation was not affected by treatment (Table 4). Following the expected normal development of the immune response, IgG titres after first inoculation were 1:2 in three birds, but absent in all the others. After the second inoculation, IgG titres were 1:2 to 1:8 in 12 birds but remained absent in 15 birds. Only in control group (T9) all broilers presented IgG titres against SRBC; contrasting, in all treated groups (T1–T8) the proportion of birds showing IgG titres varied between none and two subjects. Apart from one bird in T1, all other birds produced detectable IgM against xenogeneic erythrocytes after the first inoculation. After the second inoculation, all broilers (except in one bird in T8) produced detectable IgM titres, which varied from 1:2 to 1:16 (Table 4).

Both the length of treatment and the dietary level of the mixture significantly influenced the final weight of broilers ($P = 0.037$ and $P = 0.015$, respectively). The chickens' BW median was lower when Probio Enzyme mixture was supplied to broilers, whether in short or long treatments. Further, BW decreased with increasing amounts of mixture, particularly when its concentration was higher than 250 g/ton ($P = 0.031$; Table 5). All the treatment groups showed lower BW compared to control; only the birds from T1 presented a BW similar to those of control. The length of treatment did not affect the absolute or the relative organ weight evaluated (Table 5). However, the amount of the Probio Enzyme mixture affected the relative spleen weight ($P = 0.022$), which was slightly lower when the mixture concentrations were 250 or 1000 g/ton (Table 5). The treatment group significantly affected the absolute liver weight ($P = 0.002$), which was considerably higher in the control group compared with all the treated groups (Table 5). However, these differences were not transposed to the relative liver weight. The Kruskal-Wallis test suggests that birds from T9 and T1 were heavier. It appeared that the supplement ingested by these chickens at low doses for only 14 days did not affect BW; however, at higher doses for a longer period, the product could not be associated with improved BW in the applied test design. The dietary treatment also influenced the absolute and relative spleen weight ($P = 0.003$ and $P = 0.001$, respectively). The median absolute

Table 4 Immune response after inoculation of sheep red blood cell in broilers. Values in the table represent the median [mean rank]

Treatments		Immunoglobulins titres 14 days after 1st inoculation			Immunoglobulins titres 14 days after 2nd inoculation		
		Total Ig	IgG	IgM	Total Ig	IgG	IgM
Length (day)	Control	2 [16.67]	0 [12.00]	2 [18.50]	3 [19.00]	1 [20.50]	2 [15.67]
	Short (14)	1.5 [15.38]	0 [16.50]	1 [13.75]	2 [11.50]	0 [12.17]	2 [12.17]
	Long (42)	1 [11.96]	0 [12.00]	1 [13.12]	2.5 [15.25]	0 [14.21]	2 [15.42]
<i>P</i> value		0.433	0.059	0.513	0.232	0.181	0.507
Probio Enzyme (g/ton)	0	2 [16.67]	0 [12.00]	2 [18.50]	3 [19.00]	1 [20.50]	2 [15.67]
	250	1.5 [14.50]	0 [12.00]	1.5 [15.25]	2 [11.50]	0 [8.00]	2 [14.83]
	500	1.5 [15.33]	0 [16.50]	1 [13.25]	2 [13.42]	0 [13.25]	2 [13.25]
	750	1 [14.33]	0 [14.25]	1 [15.00]	3 [16.75]	1 [16.33]	2.5 [16.42]
	1000	1 [10.87]	0 [14.25]	1 [10.25]	2 [11.83]	0.5 [15.17]	2 [10.67]
<i>P</i> value		0.810	0.540	0.542	0.516	0.096	0.700
T1		1 [12.87]	0 [12.00] ^{a,b}	1 [13.50]	2 [11.17] ^a	0 [8.00] ^{a,b}	2 [14.00]
T2		2 [16.67]	1 [21.00] ^{b,c}	1 [11.50]	2 [6.83] ^{a,b}	0 [8.00] ^a	2 [9.50]
T3		1 [15.33]	1 [16.50] ^a	1 [15.00]	3 [16.17] ^a	1 [16.33] ^a	2 [15.67]
T4		2 [16.67]	1 [16.50] ^a	1 [15.00]	2 [11.83] ^a	1 [16.33] ^a	2 [9.50]
T5		2 [16.17]	0 [12.00] ^c	2 [17.00]	2 [11.83] ^a	0 [8.00] ^{a,c}	2 [15.67]
T6		1 [13.33]	0 [12.00] ^a	1 [15.00]	5 [10.00] ^a	1 [18.50] ^a	2 [17.00]
T7		1 [13.33]	0 [12.00] ^a	1 [15.00]	3 [17.33] ^a	1 [16.33] ^a	3 [17.17]
T8		0 [5.00]	0 [12.00] ^a	0 [5.50]	2 [11.83] ^a	0 [14.00] ^a	2 [11.83]
T9 (Control)		2 [16.67]	0 [12.00] ^a	2 [18.50]	3 [19.00] ^{a,b}	1 [20.50] ^{a,b,c}	2 [15.67]
<i>P</i> -value		0.147	0.005	0.114	0.044	0.003	0.407

Different superscripts within columns differ significantly at $P < 0.05$

spleen weight was higher in control than treated groups, and the lowest median values were recorded in broilers treated with 250 g/ton of Probio Enzyme mixture (T1 and T5). The median of relative spleen weight was higher in T2 and T7, which were close to the control treatment (T9). The thymus absolute or relative weight did not differ between dietary groups. However, in broilers fed Probio Enzyme mixture showed slightly higher medians compared with control, particularly when fed 500 g/ton or 750 g/ton, respectively.

Discussion

This study investigated the effects of treatment duration and dose of a probiotics and enzymes mixture (Probio Enzyme, XVT GmbH, Germany) on the immune response and weight of immune organs in broilers chickens. The Probio Enzyme treatment did not affect the antibody responses to NDV or AI vaccination, although in general a decrease in antibody production at day 33 was found compared to day 42, in either the short and long treatments. These results agree with previous report by Talebi et al. (2008) that found a similar gradual increase in the response to the vaccines between days 28 and 42, even if the authors failed to obtain differences between treated and control groups. Similar results have been

also recently reported by Sadeghi et al. (2015), who reported that the dietary inclusion of a probiotic-enzyme mixture had no significant effect on the immune parameters of chickens, but improving the immune response in birds when challenged with a pathogen. However, contrasting reports exist on the probiotics influence over the antibody production (Kabir et al. 2004; Haghighi et al. 2005; Midilli et al. 2008), which rose the hypothesis that in part its effects may be mediated by improved local immune defences (Kabir 2009) and by increased production of modulatory immune molecules, such as interleukins (Zhang et al. 2016).

In the present study, the immune challenged was obtained through the double inoculation of 5% sheep red blood cells. After the second inoculation, groups treated shortly with 750 g/ton of the mixture or long-treated with 500 or 750 g/ton of the Probio Enzyme mixture presented increased or equal total immunoglobulins titres than controls, which were mostly related to changes in IgG production. Similar results were reported by Khaksefidi and Ghoorchi (2006) and by Kabir et al. (2004). In a different approach, testing the oxidative burst of heterophils and monocytes, Stringfellow et al. (2011) found a favourable influence of a probiotic containing lactic acid producing bacteria in the immune response of broilers.

It is believed that, by acting locally regulating the intestinal biota, probiotics improve the growth performance of broiler

Table 5 Effects of Probio Enzyme on the defeathered weight and absolute and relative weight of thymus, liver, spleen and bursa of Fabricius in broilers. Values represent the median [mean rank]

Treatments	Defeathered weight (g)			Thymus (g)		Liver (g)		Spleen (g)		Bursa of Fabricius (g)	
		Absolute	Relative	Absolute	Relative	Absolute	Relative	Absolute	Relative	Absolute	Relative
Length (day)	Control	2300 [25.00] ^a	0.17 [11.00]	78.47 [23.67]	3.40 [21.00]	3.48 [20.83]	0.15 [18.00]	3.01 [17.67]	0.13 [11.67]		
	Short (14)	1911 [13.21] ^b	0.23 [14.42]	50.67 [13.25]	2.82 [13.42]	2.27 [13.67]	0.13 [13.50]	2.65 [14.17]	0.14 [14.58]		
	Long (42)	1864 [12.04] ^b	0.22 [14.33]	50.09 [12.33]	2.78 [12.83]	2.30 [12.63]	0.13 [13.50]	2.56 [12.92]	0.14 [14.00]		
<i>P</i> value		0.037	0.786	0.079	0.265	0.271	0.651	0.647	0.850		
ProbioEnzyme (g/ton)	0	2300 [25.00] ^{a,b,c}	0.17 [11.00]	78.47 [23.67]	3.40 [21.00]	3.48 [20.83]	0.15 [18.00] ^{a,b}	3.01 [17.67]	0.13 [11.67]		
	250	1942 [18.42] ^a	0.17 [10.33]	54.51 [15.67]	2.75 [13.50]	2.04 [6.83]	0.10 [5.33] ^{a,b,c,d}	2.67 [15.75]	0.14 [14.50]		
	500	1849 [11.58] ^{a,b}	0.25 [18.33]	50.33 [12.33]	2.69 [12.50]	2.84 [18.00]	0.15 [18.17] ^{a,c}	2.65 [15.42]	0.14 [16.67]		
	750	1750 [7.50] ^a	0.25 [15.17]	50.09 [10.83]	2.88 [13.67]	2.63 [15.67]	0.15 [18.17] ^{a,d}	2.43 [12.00]	0.13 [13.50]		
	1000	1885 [13.00] ^{a,c}	0.22 [13.67]	50.37 [12.33]	2.78 [12.83]	2.22 [12.08]	0.12 [12.33] ^a	2.82 [11.00]	0.13 [12.50]		
<i>P</i> value		0.015	0.457	0.185	0.607	0.055	0.022	0.669	0.878		
T1		2082 [22.67] ^a	0.17 [12.00]	61.80 [18.00] ^a	2.92 [14.33]	2.18 [6.83] ^{a,e,f}	0.10 [4.67] ^{a,b,c,d,e}	2.72 [17.17]	0.14 [14.33]		
T2		1902 [13.83] ^{a,b}	0.24 [18.67]	53.89 [15.67] ^a	2.75 [14.67]	2.97 [22.33] ^{a,b,c,d}	0.16 [21.33] ^{a,f,g}	2.62 [12.67]	0.14 [12.33]		
T3		1770 [8.33] ^{a,c}	0.30 [16.00]	51.08 [13.33] ^a	2.89 [15.00]	2.50 [13.83] ^{a,b,c}	0.14 [15.33] ^{a,b}	2.95 [14.00]	0.15 [16.33]		
T4		1734 [8.00] ^{a,d}	0.21 [11.00]	43.81 [6.00] ^{a,b}	2.65 [9.67]	2.26 [11.67] ^a	0.13 [12.67] ^{a,c,f}	2.39 [12.83]	0.14 [15.33]		
T5		1888 [14.17] ^{a,e}	0.16 [8.67]	47.22 [13.33] ^a	2.59 [12.67]	1.89 [6.83] ^{a,d}	0.10 [6.00] ^{a,g,h}	2.61 [14.33]	0.13 [14.67]		
T6		1796 [9.35] ^{a,f}	0.27 [18.00]	47.09 [9.00] ^{a,c}	2.63 [10.33]	2.58 [13.67] ^a	0.14 [15.00] ^a	2.72 [18.17]	0.15 [21.00]		
T7		1730 [6.67] ^{a,g}	0.21 [14.33]	49.74 [8.33] ^{a,d}	2.88 [12.33]	2.75 [17.50] ^{a,e}	0.16 [21.00] ^{a,d,h}	1.69 [10.00]	0.10 [10.67]		
T8		1911 [18.00] ^{a,f,h}	0.23 [16.33]	55.64 [18.67] ^{a,c,d,e}	2.86 [16.00]	1.91 [12.50] ^a	0.10 [12.00] ^a	2.05 [9.17]	0.11 [9.67]		
T9 (Control)		2300 [25.00] ^{b,c,d,g,h}	0.17 [11.00]	78.47 [23.67] ^{a,b,c,d,e}	3.40 [21.00]	3.48 [20.83] ^{a,f}	0.15 [18.00] ^{a,e}	3.01 [17.67]	0.13 [11.67]		
<i>P</i> value		0.031	0.303	0.002	0.354	0.003	0.001	0.423	0.325		

Different superscripts within columns differ significantly at $P < 0.05$

chickens and reduce flock mortality. However, information on the advantages of the use of probiotics on growth performance is still contradictory. In the current study, most of treated groups showed significantly lower BW than control, with exception of T1, contrasting to those reports that failed to demonstrate that probiotics may improve growth (Haghighi et al. 2005; Lee et al. 2010). These differences may be related with the constitution of the probiotics mixture, the route of administration and even with the doses tested (Gaggia et al. 2010), making difficult a net comparison of the results.

The present study also showed that the absolute or the relative weight of thymus and bursa of Fabricius remained unaffected by treatment, alike it was reported previously by others (Awad et al. 2009), suggesting therefore that the use of the Probio Enzyme mixture should not lower the immune activity based in the central lymphoid organs. The probiotic mixture used in the current study affected both the absolute and the relative spleen weight; it was in general lower in groups treated with the lowest and highest mixture doses, while in the intermediary amounts (500 and 750 g/ton), the values were identical to those found in controls. Although these findings contrast with those of Naseem et al. (2012), they support the hypothesis of an immune modulatory effect of probiotics on antibody production and an improved avian immune activity. The spleen is related with the final stages of lymphocyte differentiation and the production of memory cells for the secondary immune responses, therefore playing a crucial role in disease resistance (John 1994). Due to our experiment design, no age- or environmental-related effects are expected; hence, the differences in the spleen weight are attributed to the combined effect of the Probio Enzyme mixture and the recent immune challenges.

In conclusion, birds' groups treated with the Probio Enzyme mixture showed in general a satisfactory immune response compared with control, despite the wide variation found in particular after the first vaccine challenge against AI. The length of treatment and the dose of the probiotic mixture modulated differently the immune response and the weight of the spleen, while unaffected the weight of the central lymphoid organs. Considering all the parameters tested, the most promising combinations of length and amount of Probio Enzyme mixture were represented by short $\times$ 500 g/ton (T1) and the long $\times$ 500 g/ton (T6) or the long $\times$ 750 g/ton (T7). However, additional studies with larger number of animals would be desirable to validate these findings for the industry.

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Compliance with ethical standards

Ethic rules The procedures have been approved by authors' Institutional Ethics Committee, and care was taken to minimize the number of animals used.

Conflict of interest The authors declare that they have no conflict of interest.

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