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EFFECT OF DIETARY SUPPLEMENTATION OF SPIRULINA (*ARTHROSPIRA PLATENSIS*) AND THYME (*THYMUS VULGARIS*) ON SERUM BIOCHEMISTRY, IMMUNE RESPONSE AND ANTIOXIDANT STATUS OF RABBITS*

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Abstract

Growing rabbits' (42 rabbits/group, 3 rabbits/cage, 14 cages/treatment) diet was supplemented with 5% Spirulina (*Arthrospira platensis*) and 3% thyme (*Thymus vulgaris L.*) powder single (S or T) and in combination (ST) between 35 and 77 days of age. On day 0 (weaning at 35 days of age) 14 rabbits were vaccinated with 100 µg/animal ovalbumin to provoke immune response. Blood samples were taken on days 0, 14, 28 and 42 of the experimental period. Sampling dates significantly influenced total protein, albumin, glucose, cholesterol, urea, creatinine concentration and enzyme (AST, ALT, GGT) activities, with a significant age × diet interaction in the case of TP and CREA. There was a significant increase in ALT (+45 and 74%) and GGT (+87 and 102%) activity after immunisation. While Spirulina and thyme significantly ameliorated the rise in AST activity, their effect was inefficient in the case of GGT. Spirulina, both single and in combination showed a tendency in higher IgG level as compared to control ($P<0.05$). No significant effect of sampling date or treatment on phagocytic activity or secretory IgA was demonstrable ($P>0.05$). Higher MDA concentration was measured in the red blood cells of S, T and ST animals, while no other significant diet effect on the antioxidant parameters was detected, however, significant sampling date × diet interaction was found in the case of GPx activity. Plasma GGT (increase by 19–66%) was inversely associated with GSH (decrease by 66–113%) between days 0 to 42 of the experimental period ($r=–0.57$, $P<0.05$). It can be concluded that Spirulina supplementation alone resulted in higher IgG production, but none of the phytobiotics, at the dose used, affected significantly the antioxidant status of blood.

Key words: Spirulina, thyme, serum biochemistry, immune response, antioxidant status

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Antibiotics have been widely used in animal production for decades worldwide. From 2006 the use of antibiotics and related drugs as growth promoting feed additives to livestock has been banned within the European Union because of the risks to human health. Along with the first restrictions in the use of in-feed antibiotics in the late 1990s, intensive research has been started to develop possible alternatives for them. The 'pro-' and 'prebiotic' approach is steadily growing with the strategy to improve immune response of animals and increase resistance against diseases by feed additives which do not pose any food safety risk. All feeding strategies aiming to improve the immune response and resistance can result in more robust animals with better production and survival characteristics. Robustness in rabbits confront with more success in different environmental challenges (e.g. infection, weaning).

Spirulina (now named *Arthrospira platensis*) is a microscopic and filamentous cyanobacterium (blue-green alga) which is rich in protein, essential amino acids, vitamins, minerals and essential fatty acids (Campanella et al., 1999; Cheong et al., 2010; Jafari et al., 2014). It has been used as human food supplement for over 20 years, but its use as an animal feed supplement has been recommended and tested only in the last few years. The immunomodulatory effect of Spirulina has been thoroughly studied in many species, i.e. in catfish, chicken, mice, rats and humans, while the antioxidant effect was studied mainly by *in vitro* experiments and in laboratory rodents (mice and rats) as summarised by Belay (2002). In rabbits, Spirulina supplementation on performance parameters and apparent digestibility of nutrients (Peiretti and Meineri, 2008), meat quality and fatty acid composition of meat (Peiretti and Meineri, 2011), oxidative stress (Kim et al., 2010) and atherosclerosis (Cheong et al., 2010), as well as different effects in rabbits fed high fat diets has been studied up to now (Meineri et al., 2009; Peiretti and Meineri, 2009).

In the last decades, herbal extracts as feed additives have also been widely used as alternatives to antibiotics because of their growth-promoting effects. Thyme has been reported for its antimicrobial and antioxidant properties (Dorman and Deans, 2000). Among livestock herb extracts mixtures including thyme have been studied in pigs (Namkung et al., 2004; Yan et al., 2011), turkeys (Mikulski et al., 2008) and broilers (a mixture of dried garlic and thyme by Mohebbifar and Torki, 2011, aqueous extract of thyme by Rahimi et al., 2011, thymol and thyme oil by Hoffmann-Pennesi and Wu, 2010). Thyme powder alone was used as feed additive to laying hens (Bölükbasi and Erhan, 2007) and thyme leaves to broilers (Ocak, 2008).

The main purpose of the present study was to evaluate the effect of supplementation of the growing rabbits' diet with Spirulina (5%) and thyme powder (3%) single or in combination on blood biochemistry, certain parameters of the immune response and antioxidant status.

Material and methods

Experimental animals and housing

Individually marked weaned Pannon White rabbits (35 days of age, 952±81 g) were randomly sorted into four groups (42 rabbits/group, 3 rabbits/cage, 14 cages/

treatment) according to diet. Animals were housed in two-level wire mesh cages (3 animals per cage, 0.61×0.32 m) in a closed building. Average temperature ranged from 15 to 18°C, and the photoperiod was 16L:8D.

The research protocol was reviewed by the Animal Use and Care Administrative Advisory Committee and approved by the Agricultural Administrative Authority (Protocol No. SOI/31/254-3/2013).

Experimental diets

Rabbits were fed an isonitrogenous (170–176 g/kg), isoenergetic (16.3–16.5 MJ/kg gross energy), non-medicated basal diet, which did not include coccidiostat. The control diet (C) was formulated without supplementation, whereas the other diets were supplemented with 5% Spirulina (*Arthrospira platensis*) powder (S), or 3% thyme (*Thymus vulgaris L.*) powder (T), or both ingredients (ST), respectively, throughout 42 days (35 to 77 days of age). Diets and drinking water were provided *ad libitum*. Soybean meal represented the main ingredient that has been substituted with Spirulina, while thyme was in substitution of alfalfa meal. Spirulina and thyme powder were purchased from a commercial supplier. Chemical composition of the supplements, ingredients and nutrient content of the experimental diets are summarized in Tables 1 and 2.

Table 1. Chemical composition of Spirulina (*Arthrospira platensis)* and thyme (*Thymus vulgaris*) (g/kg)

Chemical composition	*Arthrospira platensis*	*Thymus vulgaris*
Dry matter	944.9	889.5
Crude protein	658.1	52.3
Crude fat	8.6	31.9
Crude fibre	nd[1]	181.5
Ash	65.1	65.9
Starch	35.6	58.4
Natural Detergent Fibre	2.4	298.1
Acid Detergent Fibre	4.8	209.6
Acid Detergent Lignin	0.6	68.1
Ca	2.2	13.6
P	9.2	0.7
Ca/P	0.2	18.7
Gross energy (GE) (MJ/kg)	19.5	15.7

[1] Not detected.

Analyses of Spirulina and thyme supplements and the experimental diets were carried out in duplicate using AOAC (2000) methods in order to determine the concentrations of DM (934.01), crude protein (CP; 2001.11), crude fibre (CF; 978.10), ash (967.05) and starch (amyloglucosidase-α-amylase method, 996.11). Ether extract (EE) was determined after acid-hydrolysis (EC, 1998). Neutral detergent fi-

bre (NDF, without sodium sulphite), acid detergent fibre (ADF), and acid detergent lignin (ADL) were analysed according to Mertens (2002), AOAC (2000, procedure 973.187) and Van Soest et al. (1991), respectively using the sequential procedure and the filter bag system (Ankom Technology, New York). Gross energy (GE) was measured with an adiabatic bomb calorimeter (ISO, 1998). Mineral analyses (Ca and P) were performed by ICP-OES (Spectro Ciros Vision EOP) after microwave digestion (AOAC 2000, 999.10).

Table 2. Ingredients and chemical composition of the experimental diets (g/kg)

	C	S	T	ST
Ingredients				
Spirulina	-	50	-	50
thyme	-	-	30	30
soybean meal 46%	130	55	140	60
dehydrated alfalfa meal	400	397	370	397
barley meal	247	262	237	262
wheat straw (Faser-mix)	120	110	120	90
dried apple pomace	40	40	40	40
fat powder (40%)	35	35	35	35
monocalcium phosphate	3	3	3	3
salt (NaCl)	5	5	5	5
methionine – DL	1	1	1	1
L-Lysine HCL	4	6	4	6
vitamin-mineral premix[1]	5	5	5	5
zeolit press	10	30	10	15
Analysed chemical composition				
dry matter	896	898	898	896
crude protein	176	170	175	172
crude fat	25	26	27	28
crude fibre	160	162	157	158
starch	163	181	170	178
ash	86	75	84	77
Natural Detergent Fibre	323	316	314	301
Acid Detergent Fibre	212	205	208	195
Acid Detergent Lignin	53	45	53	46
Gross energy (MJ/kg)	163	165	164	164

[1]Premix provided per kg of complete diet: vitamin A, 3.6 mg; vitamin D_3, 25 µg; vitamin E acetate, 50 mg; vitamin K_3, 2 mg; biotin, 0.1 mg; thiamin, 2 mg; riboflavin, 4 mg; vitamin B_6, 2 mg; vitamin B_{12}, 0.1 mg; niacin, 40 mg; pantothenic acid, 12 mg; folic acid, 1 mg; choline chloride, 300 mg; iron, 100 mg; copper, 20 mg; manganese, 50 mg; cobalt, 2 mg; iodine, 1 mg; zinc, 100 mg; selenium, 0.1 mg.

Vaccination and samplings

At 35 days of age (day 0) all rabbits were injected intraperitoneally with 100 µg/animal ovalbumin (Sigma-Aldrich, Hungary), dissolved in 400 µl phosphate buff-

ered saline (PBS, Sigma-Aldrich, Hungary) and 400 µl incomplete Freund's adjuvant (Sigma-Aldrich, Hungary). Vaccination was repeated on day 14 of the experiment with the same methodology.

Blood samples were taken before vaccination (35 days of age, day 0) and on days 14, 28 and 42 of the experimental period, respectively. Plasma was obtained by centrifugation (Janetzky T23, VEB, Leipzig, Germany) at 3000 rpm for 15 minutes, and used for immunological and antioxidant measurements. Parameters of the antioxidant status were determined also from red blood cell (RBC) haemolysate, which was prepared by adding 900 µl sterile destilled water to 100 µl RBC. Serum was used for the determination of clinical biochemical parameters.

On day 0 six randomly selected rabbits representing the initial condition (no treatment), while on days 14, 28 and 42 of the experimental period six healthy animals from each group (1 animal/cage) were also randomly selected and slaughtered at 11:00 am. A 10 cm long section of the distal ileum (before the ileocaecal junction) was separated and digesta collected to determine the secretory immunoglobulin A (sIgA) content.

Serum clinical biochemistry

The concentration of total protein (TP), albumin (ALB), glucose (G), total cholesterol (CHOL), urea, and the activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma-glutamyl transferase (GGT) were determined in a professional veterinary laboratory (Vet-med Laboratory, Budapest, Hungary), using Roche Hitachi 912 Chemistry Analyser (Hitachi, Tokyo, Japan) with commercial diagnostic kits (Diagnosticum, Budapest, Hungary).

Determination of immune parameters

Phagocytic activity (PA) was measured by a standard method (Simpson et al., 1979) with modifications as follows: white blood cells were separated from heparinised blood and resuspended in Eagle's Minimum Essential Medium (EMEM, Sigma-Aldrich, Hungary) supplemented with 5% fetal bovine serum (Lonza, Switzerland) and seeded to 6 well tissue culture plates. Cells were incubated in a humidified atmosphere of 5% CO_2 for 2 hours at 37 °C to allow attachment of adherent cells. Non-adherent cells were removed by washing 3 times with phosphate buffered saline (PBS). PA was determined with Congo-red stained yeast cells by incubating overnight at 37°C. Phagocytic ability was expressed as percentage of phagocytic cells quantified from 100 cells observed under a microscope.

For specific immunoglobulin G (IgG) detection microtiter plates (Sigma-Aldrich, Hungary) were coated with 100 µl of ovalbumin solution (100 µg/ml ovalbumin in PBS) per well, incubated overnight at 4°C in a humidified environment. Excess protein was removed by washing with 3x150 µl PBS-Tween 20 (Sigma-Aldrich, Hungary). Plasma samples were twofold serially diluted in the washing solution, starting with 1:50, in a separate plate. Aliquots of 100 µl of each dilution (1:50 and 1:100) were transferred to the microtitre plate (two wells for each dilution) and incubated for 1 hour at room temperature. Wells were washed again 3 times with PBS-Tween and anti-rabbit IgG-HRP conjugate (Sigma-Aldrich, 1:10000) was added to each

well, followed by incubation at room temperature for 1 hour. The wash step was then repeated and tetramethyl benzidine substrate (1 mg/ml, Sigma Aldrich) was added.

Total secretory IgA (sIgA) from intestinal contents was measured using a quantitative Rabbit IgA ELISA kit (Kamiya Biomedical Co., USA) as described by the manufacturer. Intestinal contents, 1 gram each, were suspended in 1 ml PBS and clarified by centrifugation at 5000 x g. Supernatant fluid was diluted 1:100 using the diluent buffer of the kit and applied as recommended. Quantification of IgA content was based on comparison of the optical density of each sample and the calibration curve obtained for the standards of the kit.

Measurement of antioxidant parameters

Antioxidant parameters were measured in blood plasma and red blood cell (1:9 v/v) haemolysate. Glutathione peroxidase (GPx) activity was measured using GSH and cumene hydroperoxide as co-substrates in an end-point direct assay following the system of Lawrence and Burk (1978). The enzyme activity was expressed as nmol glutathione (GSH) oxidation per minute at 25°C. GSH content was determined based on the colour complex formation of glutathione with 5,5'-dithiobis(2-nitrobenzoic acid) (Sedlak and Lindsay, 1968), while malondialdehyde (MDA) concentration based on the colour complex formation of MDA with 2-thiobarbituric acid in an acidic environment at high temperature (Placer et al., 1966).

GPx activity and GSH content was calculated to protein content of blood plasma and/or red blood cell haemolysate, which was measured by the biuret method (Weichselbaum, 1948).

Statistical analysis

Statistical analysis of the data obtained was carried out by the SPSS for Windows statistical software package using the version 11.5 (2002). Effect of treatment (diet), age and their interaction was analysed by the following general linear model:

$$y_{ijk} = \mu + T_i + A_j + TA_{ij} + e_{ijk,}$$

where:

μ = mean,
T_i = effect of treatment (diet),
A_j = effect of age,
TA_{ij} = interaction of treatment and age,
e_{ijk} = random error.

The significance of between groups differences was tested by the LSD post hoc test.

When a significant ($P<0.05$) age × treatment interaction occurred, data were further subjected to two types of statistical analyses: within the same age (among the four diets) and within the same treatment (among sampling dates).

Results

Serum biochemistry

All serum biochemical parameters were within the reference range (Table 3) as was reported by Harcourt-Brown (2002), except glucose concentration, which was elevated by 4–40% in most cases. Sampling dates significantly influenced all of the measured parameters, with a significant age × diet interaction in the case of TP and CREA (Figures 1 and 2). TP and CREA concentrations increased with time, except in animals fed with Spirulina supplemented diet, where there was an enhancement between days 14 and 28, while a slight and not significant decrease thereafter, between days 28 and 42. ALB concentration and ALT activity increased after the second vaccination, while GGT activity was higher already on day 14 compared to the starting point (day 0) and remained unchanged thereafter. CHOL concentration decreased between days 0 to 14 and 14 to 28, respectively.

The single administration of Spirulina (S) and thyme (T) resulted in smaller ($P=0.031$) ALT activity as compared to C and ST. In S rabbits TP and CREA levels were higher ($P=0.004$ and 0.005, respectively), while ALB concentration was the smallest in T group ($P=0.08$) as compared to the other three treatments. No significant response to the treatments manifested in the other biochemical parameters examined.

Immune parameters

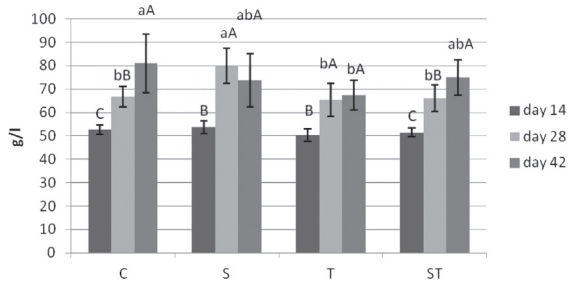
Ovalbumin specific IgG in blood plasma turned up after the first vaccination, and its level increased significantly ($P<0.05$) after the second immunisation (day 14) (Table 4). Spirulina, both single and in combination, resulted in higher IgG level as compared to control ($P<0.05$).

No significant effect of sampling date or treatment on phagocytic activity or secretory IgA and also no interaction between treatments and sampling dates were demonstrable ($P>0.05$).

Antioxidant parameters

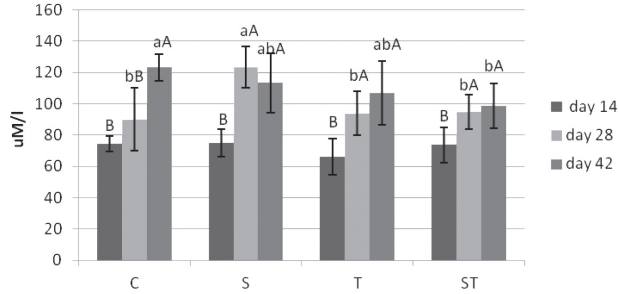
GSH and MDA concentration measured in the red blood cell haemolysate increased ($P<0.001$ and $P=0.021$, respectively) after the second immunisation, i.e. between days 14 and 28 (Table 5). Concentrations in the blood plasma slightly differed from the results of the red blood cells, namely GSH decreased between days 0 to 14 and then from day 14 to 28 ($P<0.001$). MDA concentration was less in the plasma on day 14 as compared to the other sampling dates ($P=0.013$).

Higher MDA concentration was measured in the red blood cells of animals fed with Spirulina and thyme supplemented diets compared to controls, while no other significant effect on the antioxidant parameters was detected. However, significant sampling date × diet interaction was found in the case of plasma GPx activity (Figure 3), which was transitionally smaller on day 28 as compared to data of day 14 and 42 in all groups, except in ST animals, where no difference between days 28 and 42 was observed.



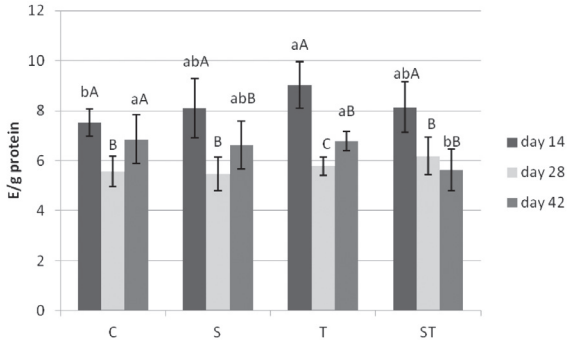
Different superscripts indicate significant differences (P<0.05) between a, b dietary treatments and A, B, C sampling dates.

Figure 1. Effect of sampling date and dietary treatments on blood total protein concentration in weaned rabbits



Different superscripts indicate significant differences (P<0.05) between a, b dietary treatments and A, B sampling dates.

Figure 2. Effect of sampling date and dietary treatments on blood creatinine concentration in weaned rabbits



Different superscripts indicate significant differences (P<0.05) between a, b dietary treatments and A, B, C sampling dates.

Figure 3. Effect of sampling date and dietary treatments on blood plasma glutathione peroxidase activity in weaned rabbits

Table 3. Effect of Spirulina and thyme and their combination on some blood biochemical parameters of growing rabbits

	Sampling (day of the experiment)[1]				Diet[1]				P value			S.E.
	0 ↓	14 ↓	28	42	C	S	T	ST	Sampling	Diet	Sampling × diet	
TP[2]	58.7 A	61.9 B	69.5 C	74.3 D	60.7 a	69.0 b	60.9 a	64.1 a	<0.001	0.005	0.013	1.4
ALB[2]	41.3 A	36.3 B	44.4 C	46.0 C	42.5 a	43.7 a	40.3 bc	42.1 ac	<0.001	0.018	0.060	0.6
G[3]	12.22 A	7.78 B	9.30 C	9.35 C	9.92	9.24	8.10	8.73	<0.001	0.063	0.075	0.21
CHOL[3]	2.22 A	2.02 B	1.78 C	1.64 C	1.90	1.99	1.66	1.82	0.002	0.107	0.055	0.05
Urea[3]	6.67 A	5.40 B	8.33 C	9.45 D	7.79	7.65	7.21	7.87	<0.001	0.193	0.152	0.25
CREA[4]	78.3 A	72.3 A	100.6 B	110.5 C	91.5 a	103.9 b	88.9 a	89.1 a	<0.001	0.004	0.005	2.5
AST[5]	40.9 A	31.1 BC	27.3 B	34.3 AC	33.8	31.4	28.7	32.1	0.018	0.714	0.274	1.1
ALT[5]	14.3 A	16.5 A	23.9 B	28.7 C	23.6 a	20.8 b	21.5 b	23.2 a	<0.001	0.031	0.194	1.0
GGT[5]	3.93 A	7.36 B	7.18 B	7.94 B	6.89	7.54	7.58	6.97	0.012	0.754	0.355	0.31

[1] n=24, ↓=vaccination, C=control, S=Spirulina, T=thyme, ST=Spirulina+thyme, S.E.=standard error.
[2] g/l. [3] mM/l. [4] µM/l. [5] IU/l.
Different letters indicate significant differences (P<0.05) between A, B, C, D sampling dates and a, b dietary treatments.

Table 4. Effect of Spirulina and thyme and their combination on certain immunological parameters of growing rabbits

Parameter	Sampling (day of the experiment)[1]				Diet[1]				P value			S.E.
	0 ↓	14 ↓	28	42	C	S	T	ST	Sampling	Diet	Sampling × diet	
IgG (mg/ml)[2]	0.00 A	4.00 B	6.93 C	7.18 C	4.85 a	6.03 bc	5.37 ac	6.28 bc	<0.001	0.048	0.427	0.25
Phagocytic activity (%)[2]	79.2	79.5	78.1	79.0	79.5	78.8	79.6	77.7	0.719	0.543	0.950	0.5
sIgA (mg/ml)[3]	3.17	3.17	3.25	3.34	3.08	3.28	3.48	3.19	0.896	0.460	0.389	0.09

[1] n=24, ↓=vaccination, C=control, S=Spirulina, T=thyme, ST=Spirulina+thyme, S.E.=standard error.
[2]Measured from blood. [3]Measured from intestinal digesta.
Different letters indicate significant differences (P<0.05) between A, B, C sampling dates and a, b, c dietary treatments.

Table 5. Effect of Spirulina and thyme and their combination on certain antioxidant parameters of growing rabbits

Parameter	Sampling (day of the experiment)[1]				Diet[1]				P value			S.E.
	0 ↓	14 ↓	28	42	C	S	T	ST	Sampling	Diet	Sampling × diet	
Red blood cell hemolysate												
GSH2	4.14 A	3.56 A	7.02 B	5.36 C	4.78	5.25	5.62	5.39	<0.001	0.383	0.540	0.20
GPx3	6.11	5.53	4.75	4.92	5.33	5.07	4.92	5.21	0.066	0.566	0.491	0.14
MDA4	16.6 AB	15.9 A	17.6B	16.7 AB	15.7 a	17.5 b	17.2 b	16.9 ab	0.021	0.012	0.146	0.3
Blood plasma												
GSH2	8.20 A	6.74 b	3.87 C	4.64 D	5.94	4.83	5.21	5.11	<0.001	0.650	0.098	0.20
GPx3	4.99 A	8.28b	5.74 A	6.47 B	6.24	6.84	7.17	6.64	<0.001	0.198	0.011	0.17
MDA4	14.9 A	12.4 b	13.7 A	14.0 A	14.3	13.3	12.6	13.4	0.013	0.071	0.347	0.2

[1] n=24, ↓=vaccination, C=control, S=Spirulina, T=thyme, ST=Spirulina+thyme, S.E.=standard error.
[2]µM/g protein. [3]E/g protein. [4]nmol/ml.
Different letters indicate significant differences (P<0.05) between A, B, C, D sampling dates and a, b dietary treatments

Close negative correlations were found between GSH concentration and GPx activity in red blood cell haemolysates (control: r=–0.95, S: r=–0.99 and ST: r=–0.71), while the opposite tendency was found in blood plasma (r=0.89, all groups counted together).

Discussion

Results presented here are part of a wide study, which involved productive performance, health status and apparent digestibility of the diets (Gerencsér et al., 2014), microbial diversity in the caecum and caecal fermentation (Vántus et al., 2012), carcass composition, vitamin B_{12} absorption into meat cuts, meat rheological traits and bone development (Dalle Zotte et al., 2014), meat fatty acid profile and its oxidative status during retail display (Dal Bosco et al., 2014).

Blood biochemical parameters refer to the health status of the animals. All values were within the normal physiological range, except glucose, which may have been influenced by the handling stress in conjunction with blood sampling.

Increase in TP concentration after vaccination could be attributable to specific antibody production (manifested in increased IgG concentration), and probably also to the synthesis of other proteins related to immune response (e.g. acute phase proteins, cytokines). The production of inflammatory proteins in the liver could lead to the temporary decrease of the albumin level after the first vaccination (Kaysen et al., 2004). Out of the four dietary treatments Spirulina increased TP by 13% as compared to control by day 28. No exact data are available if Spirulina enhances protein metabolism in the liver. In an *in vitro* trial the protein synthesis increased in the *Soleus* muscle of Spirulina fed rats, but when the effect was examined *in vivo*, no differences were observed in total protein and albumin levels between casein and Spirulina fed rats (Voltarelli and de Mello, 2008). Concerning thyme, similarly to our results Toghyani et al. (2011) reported that 0.5% dietary supplementation of thyme powder for 42 days significantly ($P<0.05$) reduced serum albumin concentration in broiler chicken, without any significant impact on albumin to globulin ratio.

The most widely published metabolic effect of Spirulina is the anticholesterolaemic effect, which could not have been supported by our results. The cause of its difference would be that plasma CHOL reducing effect of Spirulina was demonstrated in hypercholesterolaemic rats (16% inclusion, Kato et al., 1984) and rabbits (1 and 5% Spirulina inclusion, Cheong at al., 2010) fed high cholesterol diet. In the present study no hypercholesterolaemic diet was used and control animals showed normocholesterolaemic status. Meineri et al. (2009) found significant difference in the HDL-CHOL level of rabbits fed high and low fat diets, but supplementation with 1% Spirulina did not influence this effect, namely Spirulina did not protect animals from dyslipidaemia.

Plasma enzyme levels, such as AST, ALT and GGT are largely used to detect liver damage, because they are located within the cells and are released to the blood as a consequence of cellular damage. As in this experiment no hepatic injury happened,

enzyme levels remained within the physiological ranges. On the other hand there was a significant increase in ALT (+45 and 74% by days 28 and 42, respectively) and GGT (+87 and 102% by days 14 and 42, respectively) activity after immunisation. While Spirulina and thyme individually ameliorated the rise in ALT activity, their effect was inefficient in the case of GGT. Serum GGT activity is commonly used as a marker for excessive liver diseases. On the other hand a series of epidemiological studies suggested that serum GGT within its normal range might be an early and sensitive enzyme related to oxidative stress, as being a key enzyme in the catabolism of extracellular GSH (Lee et al., 2004). For example, serum and dietary antioxidant vitamins had inverse, dose-response relations to serum GGT level within its normal range. GGT is inversely associated with antioxidants (Makhlouf and Makhlouf, 2012) as it could be demonstrated in the case of plasma GGT (increase by 19–66%) and GSH (decrease by 66–113%) between days 0 to 42 of the experimental period in present study ($r=-0.57$, $P<0.05$).

Vaccination of the rabbits with ovalbumin provoked specific IgG production, which would be important in rabbit production to improve immunity in prevention of infectious diseases. Using immunostimulative feed additives is one way to improve the immunity of animals and to decrease their susceptibility to infectious disease. The immunomodulatory effect of Spirulina has been widely published, although most results originate from *in vitro* experiments and *in vivo* studies with laboratory animals, as it was summarised by Belay (2002) and Ravi et al. (2010). Hayashi et al. (1994) published detailed studies on the immunomodulatory properties of dietary Spirulina in mice, and concluded that it enhances immune function through the modulation of phagocytosis and interleukin-1 (IL-1) production. In our study no change in macrophage activity was observed, but Spirulina both in single administration and also when combined with thyme resulted in more specific IgG production.

There are some contradictory results about the effect of thyme on immune function but none of them in mammals (Sadeghi et al., 2012; Toghyani et al., 2010, 2011). In our study with rabbits supplementation of thyme powder did not influence antibody production.

Because of its high carotenoid, polyunsaturated fatty acid, vitamins E and B, and mineral, including selenium content, Spirulina is consider to prevent diseases that are associated with oxidative stress or inflammation (Deng and Chow, 2010). On the other hand this effect has been proved mainly in oxidative stress induced animals and with much higher concentration as was used in this experiment (Makhlouf and Makhlouf, 2012).

In the present study three main parameters were used as biomarkers to assess the level of oxidative stress in the organism: the level of cytotoxic and genotoxic MDA, GSH as one of the main intracellular free radical scavengers, and selenium-dependent glutathione peroxidase (GPx) which is part of the enzymatic antioxidant defence mechanism. It was expected that the vaccination-induced immune response weakened the antioxidant status of the animals, therefore dietary antioxidant supplementations having a compensatory effect. However, results of the present study were not really congruent. Vaccination influenced the antioxidant system, as shown by the slight and temporary differences in GSH content and GPx activity between initial

values and after the first and second vaccination, but supplementation with antioxidants by Spirulina and/or thyme did not have measurable effects. Antigen injection did not result in increased MDA production referring oxidative stress, presumably because glutathione peroxidase as antioxidant enzyme had the capability to reduce formation of oxygen free radicals and consequently MDA.

It can be concluded that dietary supplementation of Spirulina and thyme did not have a pronounced effect on the immune response and antioxidant status, therefore further studies should be carried out and focus on other effects, i.e. effect of concentrations and the length of supplementation. The different methodologies used in the different *in vivo* experiments to monitor changes in the immune response and antioxidant status can also be considered as a reason for the lack of consistency in the results. It is also likely that feed processing (e.g. pelleting), storage and packaging conditions may reduce the amount and/or availability of the different functional compounds, as supposed by Gerencsér et al. (2014).

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