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Effects of dietary marine microalgae *Schizochytrium limacinum* on natural humoral immunity of broiler chickens

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Abstract

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The aim of the study was to evaluate the effect of dehydrated whole cell dietary marine microalgae *Schizochytrium limacinum* on natural humoral immunity of broiler chickens, including serum lysozyme concentrations, alternative pathway of complement activation, beta (β) lysine, alfa (IFN-α) and gamma (IFN-γ) interferons. The first completely randomised experimental design included 90 (ninety) and the second – 120 (one hundred twenty) one day-old Ross 308 male broiler chickens that were obtained from a local commercial hatchery. Upon arrival, all chickens were individually weighted, wing-banded, and assigned randomly in three (Experiment 1) and fourth (Experiment 2) groups respectively, with three subgroups (replicates) of thirty birds each. They were housed in separate pens into wire type experimental cages that were placed in an environmentally controlled experimental poultry house. All experimental basal diets were formulated to meet or exceed broiler chick's nutritional requirements. The microalgae used in this study was a dehydrated, whole-cell *Schizochytrium limacinum* CCAP 4087/2, as a source of highly unsaturated fatty acids (DHA and EPA), supplemented with low and moderate doses. Water and feed were provided *ad libitum* throughout the experiments. The trials were terminated when the broiler chickens were 42 day of age. On the base of obtained results we can conclude that marine microalgae *Schizochytrium limacinum,* supplemented with low and moderate dietary doses, don't alter immune functions of tested indices in broiler chickens and even increase them after six weeks of treatment.

Keywords: marine microalgae; *Schizochytrium limacinum*; natural humoral immunity; broiler chickens

Introduction

Microalgae are unicellular, photosynthetic aquatic plants. They are introduced to poultry diets mainly as a rich source of n-3 long chain polyunsaturated fatty acids (PUFAs), including Docosahexaenoic (DHA) and Eicosapentaenoic acid (EPA), but they can also serve as a protein, microelement, vitamin and antioxidants source, as well as a pigmentation agent for skin and egg yolks. Most experiments have shown that microalgae, mainly *Spirulina* and *Chlorella* can be successfully used as a feed ingredient of poultry nutrition (Świątkiewicz et al., 2015).

It is known that *Schizochytrium* sp. dried microalgae (DRM), including *Schizochytrium limacinum,* contains oil rich in highly PUFAs as DHA and EPA (Yaguchi et al., 1997; Shwu-TzyWu et al., 2005; Chi et al., 2009). DHA is the most abundant PUFA component of the algal oil (approx. 35% w/w). DHA-rich extracted oil from *Schizochytrium* sp. is intended for use as a nutritional ingredient in foods (Hammond et al., 2001). Some scientific studies demonstrate that administration of DRM did not produce any treatment-related adverse effects in rats, rabbits, and swine (Hammond et al., 2001, 2002; Abril et al., 2003; Fedorova-Dahms et al., 2011; Schmitt et al., 2012). In this relation, investigations of productive traits and chemical composition of meat are made and was found that DHA supplementation did not cause significant changes of crude composition and sterols. On the other hand, DHA-enriched broiler's whole carcass, breast and legs contained 3.1 to 5.6 times more DHA, have lower omega 6/3 ratios, lower tocopherols content, and contained significantly more carotenoids. It was proposed that consumption of roasted DHA-rich broiler's breast and legs probably cover a significant fraction of daily protein and DHA intake, without overloading the consumer with cholesterol, and at the same time providing enough amounts of important micro constituents (Kalogeropoulos et al., 2010). Moreover, several immunomodulating compounds are also produced by this microalgae, which highlights a potential contribution to vaccine efficacy when used as a delivery vehicle (Ramos-Vega et al., 2018).

In their review Fraeye et al. (2012) discus the recent trend for feed supplementation with microalgae as a source of EPA and/or DHA concerning omega-3 PUFAs enrichment in eggs. The results of some published experiments have shown that using of marine microalgae (*Schizochytrium* sp.) in poultry nutrition could be of interest not only as a source of nutrients, but also as an alternative way of enriching of eggs and poultry meat with health-promoting long-chain n-3 PUFAs (Gladkowski et al., 2014; Trziszka et al., 2014; Park et al., 2015).

In addition, microalgae has many potential bio-functional activities (Samarakoon & Jeon, 2012). In the last decade were published several studies which regard influence of DHA and EPA on inflammatory and immune status in humans and laboratory animals. Kelley (2001) points out that supplementation of human diets with omega-3 fatty acids reduced neutrophil, monocyte, and lymphocyte functions, including the production of inflammatory mediators. Similar results reported Kew et al. (2004) and Weldon et al. (2007). In their review, Chapkin et al. (2009) describes a mechanistic model that may explain the pleiotropic anti-inflammatory and immunosuppressive properties of EPA and DHA. Authors show three mechanisms of n-3 PUFA action: (i) modulation of nuclear receptor activation, i.e., nuclear factor-κB (NF-κB) suppression; (ii) suppression of arachidonic acid-cyclooxygenase-derived eicosanoids, primarily prostaglandin E2-dependent signaling; and (iii) alteration of the plasma membrane micro-organization (lipid rafts), particularly as it relates to the function of Toll-like receptors (TLRs), and T-lymphocyte signaling molecule recruitment to the immunological synapse.

Currently, very little studies illustrate the capability of PUFAs to modulate immune activity and they provide a platform to further study immune functions of animals (Caroprese et al., 2009).

In the available literature, there is not enough information about application of *marine* derived feed ingredients in broiler nutrition and their effects on the immune system. The influence of microalgae supplementation on immune responses also is not well understood.

The aim of this study was to evaluate the effects of dehydrated whole cell dietary marine microalgae *Schizochytrium limacinum* on natural humoral immunity of broiler chickens, including serum lysozyme concentrations, alternative pathway of complement activation (APCA), beta (β) lysine, alfa (IFN-α) and gamma (IFN-γ) interferons.

Materials and Methods

Animals, Diets and Experimental Design

The study was conducted in the Poultry unit, Faculty of Agriculture, Trakia University, Stara Zagora, Bulgaria. The experimental poultry house and equipment were cleaned and disinfected before starting the experiments. The first completely randomised experimental design included 90 (ninety) and the second – 120 (one hundred twenty) one day-old Ross 308 male broiler chickens that were obtained from a local commercial hatchery. Upon arrival all chickens were individually weighted, wing-banded, and assigned randomly in three (Experiment 1) and fourth (Experiment 2) groups respectively, with three subgroups (replicates) of thirty birds each. They were housed in separate pens into wire type experimental cages that were placed in an environmentally controlled experimental poultry house. Pens were equipped with plastic feeders and drinkers. All chickens were kept under the same managerial, hygienic and environmental conditions. The rearing environment complied with the Ross breeder's recommendations (Ross Broiler Management Manual, 2009). The trials were terminated when the broiler chickens were 42 day of age. The experiments were conducted within standard ethical norms and no birds were subjected

to undue stress. The experimental protocol was approved by the Animal Experimental Committee of Trakia University, Stara Zagora, Bulgaria.

During the study, a total of three (Experiment 1) and four (Experiment 2) dietary treatments were applied. Each treatment considered of three replicates of 10 chickens per replicate.

A three-phase feeding program was used with commercial no medicated type corn-wheat-soybean based diets: starter (from 1st to 21st day); grower (from 22nd to 35th day) and finisher (from 36st to 42nd day). All experimental basal diets were formulated to meet or exceed broiler chick's nutritional requirements, as recommended by National Research Council /NRC/ (1984), in particular as recommended by Ross Broiler Management Manual (2009). The basal diets were the same for all groups (Table 1). They were mixed as a single batch to reduce diet variability after which the feed supplement was added to create the different dietary treatments. The treatments were as follows:

Experiment 1*:* Group 1 – Negative control (NC): Basal diet with no supplementation; Group 2: Basal diet with *Schizochytrium limacinum* (starter-0.5%; grower-0.25%; finisher-0.125%) and Group 3: Basal diet with *Schizochytrium limacinum* (starter-1.0%; grower-0.5%; finisher-0.25%).

Experiment 2: Group 1 – Negative control (NC): Basal diet with no supplementation; Group 2: Basal diet with *Schizochytrium limacinum* (starter-1.0%; grower-0.5%; finisher-0.25%); Group 3: Basal diet with *Schizochytrium limacinum* (starter-1.5%; grower-0.75%; finisher-0.375%); Group 4: Basal diet with *Schizochytrium limacinum* (starter-2.0%; grower-1.0%; finisher-0.5%).

The microalgae used in this study is a dehydrated, whole-cell of *Schizochytrium limacinum* CCAP 4087/2*,* which contains 64% fat, 16% DHA and 11% crude protein. They were provided by Alltech Bulgaria Ltd. All the diets did not contain any coccidiostats, antimicrobial growth promoters and prophylactics and were presented in mash form. Water and feed were provided *ad libitum* throughout the experiments.

Methods

The blood samples were bled from *v. subcutanea ulnaris* and were left to clot for 1.0 h. Then blood sera were centrifuged at 2000g for 10 min, collected in different sterile tubes, and stored at (-25Cº) until the time of investigation. Serum lysozyme concentrations were determined by method of Lie (1985); Alternative pathway of complement activation (APCA) by method of Sotirov (1986); β-lysine by method of Buharin et al. (1977); Alfa (IFN-α) and Gamma (IFN- γ) interferons by ELISA tests (Chicken IFN-α, cat. N 201-16-0003 and IFN-γ, cat. N 201-16-0017 ELISA Kits, Shanghai Sunred Biological Technology Co., Ltd, China). The obtained data were processed by one way ANOVA with fixed effects of the factor using Statistica 6.0 (StatSoft Inc.).

Table 1. Composition and nutrient content of basal diets

Dietary ingredient, % (as-is)	Diets		
	Starter	Grower	Finisher
Corn	30.00	33.00	33.34
Wheat	29.00	30.40	30.00
Soybean meal (47% CP*)	30.00	23.00	19.00
Sunflower meal (37% CP)	3.00	5.00	8.00
Sunflower oil	3.50	4.70	6.00
L-Lysine HCL (56%)	0.30	0.28	0.35
DL-Methionine (free base)	0.25	0.18	0.17
L-Threonine (free base)	0.08	0.08	0.08
Phytase®	0.01	0.01	0.01
Calcium carbonate	0.61	0.5	0.28
Dicalcium phosphate	2.20	1.85	1.80
Salt ($NaCI_2$)	0.25	0.20	0.15
Sodium bicarbonate	0.30	0.30	0.32
Vitamin-Mineral Premix**	0.50	0.50	0.50
Calculated nutrient composition.			
Metabolizable energy, Kcal/kg	3003	3121	3195
Crude Protein, %	22.00	19.70	19.00
Calcium, %	1.00	0.90	0.76
Available phosphorus, %	0.46	0.40	0.39
Methionine, %	0.58	0.49	0.48
Methionine + Cysteine, %	0.94	0.82	0.80
Lysine, %	1.32	1.14	1.12
Threonine, %	0.86	0.77	0.74
Tryptophan, %	0.25	0.22	0.21
Sodium, %	0.20	0.18	0.16

* CP – Crude Protein

** The vitamin and mineral premix contained vitamins and trace elements to meet the requirements specified by the NRC (1994). The premix provided (units/kg diet): Retinol, 3.6 μg; Cholecalciferol, 125 μg; α-tocopherol, 34 mg; Menadione, 3 mg; Thiamine, 2 mg; Riboflavin, 7 mg; Pyridoxine, 5 mg; Cobalamin, 15 μg; Nicotinic acid, 50 mg; Pantothenic acid, 15 mg; Folic acid, 1 mg; Biotin, 200 μg; Iron, 80 mg; Copper, 10 mg; Manganese, 100 mg; Cobalt, 0.5 mg; Zinc, 80 mg; Iodine, 1 mg; Selenium, 0.2 mg; Molybdenum, 0.5 mg

Results and Discussion

The results from the first experiment are presented in Table 2. The experimental data show that serum lysozyme concentrations slowly decrease to the III group with *Schizochytrium limacinum* (starter-1.0%; grower-0.5%; finisher-0.25%). APCA has the highest value in II group with *Schizochytrium limacinum* (starter-0.5%; grower-0.25%;

Table 2. Effects of dietary marine microalgae *Schizochytrium limacinun* on the values of some factors of natural humoral immunity in broiler chickens

Traits\Groups	Untreated Control	Experimental Groups	
	I group	II group	III group
Lysozyme, mg\L	0.77 ± 0.24	0.76 ± 0.15	0.64 ± 0.08
APCA (CH50)	541.25 ± 29.18	559.85 ± 25.53	515.59 ± 24.62
β-lysine, %	11.33 ± 3.14	8.82 ± 1.04	11.49 ± 1.30
IFN-α, pg/ml	71.91 ± 21.05	49.28 ± 8.50	78.33 ± 24.10
IFN-γ, pg/ml	165.72 ± 26.68	192.48 ± 30.22	149.09 ± 28.14

finisher-0.125%), but both interferons (IFN-α and IFN-γ) varies in different way. The differences at all these indices are not significant. In β-lysine the highest value is observed in III experimental group and in this case also there are no significant differences between tested groups.

Results obtained from the second experiment are presented in Table 3. As it can be seen the obtained experimental results are more expressive. Lysozyme concentrations are decreased gradually from control group to fourth group. APCA has highest value in I group, then slowly decrease in II and III groups, and is almost restored in IV group with *Schizochytrium limacinum* (starter-2.0%; grower-1.0%; finisher-0.5%). IFN-α and IFN-γ varies in similar way and had highest values in the fourth groups. Values of β-lysine vary in narrow limits and that is why no significant differences were found.

Regarding the results presented in Table 2 can be seen that no strong effect of dehydrated whole cell dietary marine microalgae *Schizochytrium limacinum* was observed. Probably this is due to the low doses applied in this case. But if carefully regard data presented in Table 3 it can be notice that there is reduction of lysozyme concentrations in II group, but in III and IV groups they are increased in parallel with increasing of the dietary doses. Variation of the other indices is similar i.e., there are limited reduction in II or III groups followed of noticeable increasing in IV group. So, it can be concluded that there is dose dependent effect between values of studied indices and applied dietary doses of *Schizochytrium limacinum*.

It is difficult to do discussion of our results because we did not find investigations which regard effect of dehydrated whole cell dietary marine microalgae *Schizochytrium limacinum* on serum lysozyme concentrations, alternative pathway of complement activation (APCA), β-lysine, and IFN-α and IFN-γ interferons. Nevertheless, we will discuss the results obtained by other authors examining effect of *Schizochytrium* sp. DRM as a source of n-3 long chain PUFAs, including DHA and EPA on some other factors of the immune system.

Generally, the relationship between *Schizochytrium* sp. immunostimulatory compounds and the immune system is not fully understood. There are some reports that DHA and EPA have proinflamatory and anti-inflammatory effects (Kelley, 2001; Ramakers et al., 2005). DHA and EPA differently modulate immune responses related to phagocytosis, activation of transcription factors and effector immune-related gene expression, which largely depends on the cell type or target species (Ramos-Vega et al., 2018).

According to Gorjão et al. (2006, 2009) DHA and EPA enhance neutrophil and macrophage phagocytosis, nitric oxide production (a pro-inflammatory mediator), and lymphocyte proliferation. On the other hand, they promote B-cell activation and antibody production, particularly enhancing mucosal IgA responses, which is relevant to protect against infectious diseases (Gurzell et al., 2013; Whelan et al., 2016).

In his review Kelley (2001) summarize that supplementation of human diets with ω-3 fatty acids reduced several aspects of neutrophil, monocyte, and lymphocyte functions, including the production of inflammatory mediators. Most of the studies have indicated reductions in these functions,

Table 3. Effects of dietary marine microalgae *Schizochytrium limacinun* on the values of some factors of natural humoral immunity in broiler chickens

Traits\Groups	Untreated control	Experimental groups		
	I group	II group	III group	IV group
Lysozyme, mg\L	1.00 ± 0.31	0.49 ± 0.14	0.71 ± 0.26	0.79 ± 0.39
APCA (CH50)	547.84 ± 27.14	526.26 ± 18.25	532.96 ± 18.01	540.87 ± 26.45
β lysine, %	22.26 ± 3.53	28.69 ± 11.72	29.07 ± 8.87	29.16 ± 8.15
IFN-α, pg/ml	101.52 ± 20.10	93.70 ± 16.60	115.12 ± 7.01	114.18 ± 26.30
IFN-γ, pg/ml	124.15 ± 46.20	131.37 ± 29.05	126.55 ± 23.30	219.96 ± 34.60

with a minimum of 1.2 g/d of supplementation with EPA and DHA for 6 weeks.

Kew at al. (2004) reported that supplementation with DHA, but not with EPA, suppresses T-lymphocyte activation but no other marker of immune function was significantly affected by either EPA or DHA. The same authors in other publication let us know that human neutrophils, monocytes, or lymphocytes do not alter their functional activity after treatment with ≤ 9.5 g ALA/d or ≤ 1.7 g EPA+DHA/d.

Weldon et al. (2007) investigated the differential effects of pure EPA and DHA on cytokine expression and nuclear factor κB (NF-κB) activation in human THP-1 monocyte-derived macrophages. They found that pretreatment with 100 μM EPA and DHA significantly decreased lipopolysaccharide-stimulated THP-1 macrophage tumor necrosis factor (TNF) α, interleukin (IL) 1β and IL-6 production. According to them a low dose (25 μM) of DHA had a better inhibitory effect than that of EPA on macrophage IL-1β and IL-6 production after LPS stimulation. They also found that EPA and DHA reduced TNF-α, IL-1β and IL-6 mRNA expression.

Vedin et al. (2008) treated Alzheimer disease patients by 1.7 g DHA and 0.6 g EPA daily and found increased plasma concentrations of DHA and EPA, which were associated with reduced release of IL-1, IL-6, and granulocyte colony-stimulating factor from peripheral blood mononuclear cells. Macalintal et al. (2013) investigated the effect of adding different levels of microalgae *Schizochytrium* sp. to broiler chicks' diets on specific humoral immunity and found that the highest antibody production to sheep red blood cells (including IgG and IgM) is observed in dose 1.0%.

Conclusion

On the base of obtained results, we can conclude that dehydrated whole cell marine microalgae *Schizochytrium limacinum* CCAP 4087/2*,* supplemented with low and moderate dietary doses, as a source of highly unsaturated fatty acids (DHA and EPA), don't alter immune functions of tested indices in broiler chickens and even increase them after six weeks of treatment.

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