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Original article

Effect of fungal solid-state fermented product enriched with gamma-linolenic acid and ß-carotene on blood biochemistry and immunology of broiler chickens

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Abstract

The aim of this study was to investigate the effect of the addition of fungal solid-state fermented product (FP) enriched with gamma-linolenic acid (GLA) and β-carotene to feed on the haematological and immunological parameters of broiler chickens. Eighty 1-day-old COBB 500 broiler chickens were divided into two groups. The control group was fed with basic diets and chickens of the experimental group received 10% addition of FP, while the amount of basic diet was reduced. FP was produced during a solid-state fermentation (SSF) process using *Umbellopsis isabellina* CCF2412 as a producer of GLA and β-carotene. After 38 days of feeding, blood samples were collected and analyzed. Lower total and LDL-cholesterol values were measured in blood samples of the experimental animals ($p<0.05$). However, the triacylglycerol content was higher in the experimental group ($p<0.05$). Significantly higher levels of hematocrit and hemoglobin, and lower eosinophil and basophil content in the experimental group were recorded ($p<0.05$). The experimental group showed higher numbers of B lymphocytes and greater phagocytic capacity ($p<0.05$). The results indicate that a fermented product produced by SSF, using the fungal strain *Umbellopsis isabellina*, is a good source of GLA and β-carotene, which can influence the biochemical, hematological and immunological parameters of broiler chickens.

Key words: broiler chicken, lipids, immunology, fermented product, blood chemistry

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Introduction

Cereal-based feed has a high n-6/n-3 ratio and, in addition, lacks a multitude of other important polyunsaturated fatty acids (PUFAs) such as gamma-linolenic acid (GLA), dihomo-gamma-linolenic acid (DGLA), arachidonic acid (AA) and docosahexaenoic acid (DHA). PUFAs have several uses in various biomedical and nutraceutical fields due to their structural and functional properties. They regulate the architecture, dynamics, phase transition, and permeability of membranes as well as the behaviour of some membrane-bound proteins as essential compounds (Wang et al. 2017). GLA (C18:3 n-6) is synthesized from linoleic acid (C18:2 n-6; LA) in a reaction catalysed by the rate-limiting enzyme Δ6-desaturase. The rate of endogenous conversion of linoleic acid to GLA is low or fails in a variety of diseases and pathological conditions, such as atopic dermatitis, diabetes neuropathy, premenstrual syndrome, and rheumatoid arthritis (Sergeant et al. 2016, Guil-Guerrero et al. 2018). The major commercially available GLA-rich oils are derived from the seeds of the evening primrose (8-10% GLA), borage seeds (24-25% GLA), and blackcurrant seeds (16-17% GLA). Since these plants cannot meet the increasing market demand for GLA-oils, attention has been focused on seeking other suitable sources of this fatty acid. There are many well-known microbial producers of various types of PUFAs. The most promising method is actually one of the oldest known cultivation processes – solid-state fermentation (SSF). Suitable strains for SSF process are filamentous fungi. Among this kingdom, the zygomycetous filamentous fungi, e.g. *Thamnidium elegans, Cunninghamella echinulata*, *Umbelopsis isabellina* and *Mortierella alpina*, are one of the greatest producers of PUFAs such as GLA or AA (Čertík et al. 2013). SSF substrates such as rice bran, wheat bran, corn meal or spent malt grains provide a suitable source of nutrients for mould growth and the production of desired PUFAs. Furthermore, these strains are able to produce more biologically active lipophilic compounds, e.g. carotenoid pigments, coenzyme Q10, sterols and even various types of hydrolytic enzymes (Bača et al. 2014, Kovalík et al. 2018).

The basis of broiler nutrition is to achieve harmonious development, growth, utility and good health. Several nutritional factors such as energy, selective amino acids, PUFAs, vitamins, minerals, etc. are known for their influence on the immune system of poultry (Collins and Moran 2001, Buyse et al. 2009). The quantity and quality of received fatty acids directly influences immune status and hence the function of the whole organism. Both maize and wheat presented in standard commercial mixture broiler feed are rich in linoleic acid (LA) but there is a lack of a significant amount of PUFAs. This is the first study focused on feeding with FP prepared by SSF enriched by gamma-linolenic acid and β-carotene.

A non-toxigenic strain of the lower filamentous fungi *Umbelopsis isabellina* that is known as a good producer of GLA and β-carotene (Klempová et al. 2013) was used for fermented product (FP) preparation. FP produced by SSF on cornmeal was added to the broiler diet at a dose of 10% instead of basal feed. The aim of the study was to investigate the effect of this addition on biochemical, hematological and immunological parameters of blood obtained from broiler chickens.

Materials and Methods

Preparation of fermented product

The strain *Umbelopsis isabellina* CCF2412 was obtained from the Culture Collection of Fungi, Charles University, Prague, Czech Republic. The culture was maintained on potato-dextrose agar (PDA; Carl Roth, Germany) at 4°C and reinoculated every 3 months. The substrate for SSF, corn meal, was obtained from Amylum Slovakia Ltd. (Boleráz, Slovak Republic). The SSF procedure was carried out according to Marcinčák et al. (2018). After fermentation, the obtained product was dried at 50°C until constant weight was achieved.

Animals, diets and management

The animal protocol for this research was approved with the consent of the State Veterinary and Food Administration of the Slovak Republic No. 3090/13-221 in the premises of the Clinic for birds, exotic and free living animals of the University of Veterinary Medicine and Pharmacy in Košice (The Slovak Republic). One-day-old COBB 500 (*Gallus gallus domesticus*) sexed male broiler chickens (n = 80) were purchased from Hydina Slovensko Ltd. (Slovak Republic) and divided into control and experimental groups (n = 40). Each group was divided in four replicates (n = 10). All chicks were supplied with diets: a starter diet during the first 10 days of fattening; a growing diet from day 11 to 28; a final diet from day 29 to 38. The main components of feed mixtures were wheat, corn, soybean meal, rapeseed cake and sunflower meal. The control group of animals was fed with basic feed mixtures (starter, grower and finisher) without supplementation of FP. Chickens of the experimental group were fed with a diet enriched with fermented product in 10% concentration from the 11th day of fattening (grower and

Table 1. Specification of mouse anti-chicken monoclonal antibodies.

Type	Fluorochrome	Clone	Isotype	Concentration	Amount/5.10^5
Anti-CD3	FITC	CT-3	IgG1 k	0.1 mg/ml	2 µl
Anti-CD4	FITC	CT-4	IgG1 k	0.5 mg/ml	2 µl
Anti-CD8a	R-PE	CT-8	IgG1 k	0.1 mg/ml	1 µl
Anti-CD45	APC	LT-40	IgM k	0.1 mg/ml	5 µl
Anti-IgM	R-PE	M-1	IgG2b k	0.1 mg/ml	1 µl

finisher diet). The broilers were reared on a deep pod (shavings). During the whole time of fattening the light and temperature regime was monitored according to the Cobb Broiler Management Guide (2013). The animals had access to water and feed *ad libitum* during fattening. In the case of both groups, neither clinical symptoms of disease nor abnormal mortality were observed during the fattening period.

Determination of fatty acids of feed

Fatty acids and β-carotene of fermented product and feed mixtures of the control and experimental groups were determined as their methyl esters by gas chromatography according to Marcinčák et al. (2018). The fatty acid methyl esters were identified by authentic standards (Supelco, Bellefonte, PA, USA) via ChemStation software B0103 (Agilent Technologies, Santa Clara, CA, USA).

Blood samples collection and Immunological examination

Blood samples were taken on the 39th day of the fattening period from the wing vein using disposable, sterile syringes and transferred to sterilized centrifuge tubes (n=12). Total cholesterol, lipid and triglyceride levels were determined from blood serum by Tietz (1995). For HDL-cholesterol the determination method was according to that described by Sugiuchi et al. (1995). Concentration of LDL-cholesterol was determined using the method according to Bachorik (1997). HDL-cholesterol was enzymatically determined with cholesterol esterase and cholesterol oxidase. Determination of aspartate-aminotransferase (AST), alkaline-phosphatase (ALP) and alanine-transaminase (ALT) was based on absorbance measurement according to Tietz (1995). A Cobas C111 biochemical analyzer (Roche diagnostics Ltd., Switzerland) was used for spectrophotometric methods.

Phagocytic activity was evaluated using a commercial kit (Phagotest, BD Biosciences, San Jose, CA, USA). Lymphocytes were phenotyped after isolation of mononuclears from heparinized blood. Blood (600 µl) diluted at a 1:1 ratio with PBS (MP Biomedicals, France) was carefully underlayered with 2 ml of lymphocyte separating medium (LSM) 1077 (PAA Laboratories GmbH, Austria) and centrifuged for 30 min at 600 x g. Lymphocytes were retrieved from the plasma-LSM interphase and subsequently washed twice with PBS before centrifugation for 5 min at 250 x g. Lymphocyte subpopulations were identified after direct staining with conjugated monoclonal antibodies (Southern Biotech, USA). Specification of the antibodies used is presented in Table 1. Blood was incubated with monoclonal antibodies for 20 min in the dark at laboratory temperature. After washing with PBS, 100 µl of PBS was added to each sample and flow cytometric analysis was performed on a six colour BD FACSCanto™ flow cytometer. BD FACS Diva™ software was used for data analysis. Position of lymphocytes was gated in flow cytometric immunophenotyping of lymphocyte (FSC) vs. side scatter (SSC) dot plot. Contaminating chicken thrombocytes were differentiated from lymphocytes based on their higher side scatter position (Bertram et al. 1998). Proportions of lymphocytes are expressed in percentages.

Total numbers of erythrocytes and leukocytes were determined by counting in the Bürker cell. Hematocrit values were determined using the micro-hematocrit method (Bertram et al. 1998). Hemoglobin values were determined spectrophotometrically. The leukogram was microscopically evaluated from Diff-Quick stained blood.

Statistical analyses

Data analysis was carried out using Graph Pad Prism 8.3 software (2019, USA). The results of each variable were expressed as mean and standard error of the mean (SEM). Student's unpaired t-test was used to evaluate the statistical significance between the control and experimental group, with a confidence interval set at 95%. The statistical significance level was set at $p<0.05$.

Table 2. Fatty acid profile of fermented product and broiler diets.

Fatty acids	DIET				
	Control			Experimental	
	FP	Grower	Finisher	Grower	Finisher
C12:0	0.000	10.652	10.948	8.697	9.016
C14:0	0.000	4.086	4.178	3.440	3.302
C16:0	16.613	12.861	14.355	14.073	14.812
C16:1 n-7	0.000	0.075	0.114	0.092	0.118
C16:1 n-9	1.495	0.369	0.683	0.715	0.863
C18:0	2.851	3.282	4.487	3.209	4.233
C18:1 n-9 (OA)	40.503	30.259	30.097	33.040	32.431
C18:1 n-7	0.000	2.007	2.104	1.655	1.891
C18:2 n-6 (LA)	31.189	30.796	28.087	29.105	28.195
C18:3 n-6 (GLA)	3.486	0.000	0.000	1.141	0.789
C18:3 n-3 (ALA)	0.751	3.053	2.773	2.526	2.375
C20:0	0.249	0.334	0.288	0.479	0.392
C20:1 n-9	0.000	0.456	0.487	0.471	0.475
C22:0	0.000	0.242	0.159	0.256	0.254
C24:0	0.000	0.178	0.143	0.240	0.203
C24:1 n-9	0.000	0.048	0.036	0.080	0.077
β-carotene (µg/g)	3.119	0.000	0.000	0.045	0.032
GLA (mg/g FP)	3.036	0.000	0.000	0.626	0.432

FP: fermented product; OA: oleic acid; LA: linoleic acid; GLA: gamma linolenic acid; ALA: alpha linolenic acid.

Table 3. Effect of fermented product on selected biochemical blood parameters (n=12).

	Control	Experimental	SEM	p-value
CHOL, mmol/l	3.45	3.27	0.126	0.05
HDL-C, mmol/l	2.28	2.29	0.122	0.92
LDL-C, mmol/l	1.88^{a}	1.72^{b}	0.067	0.05
TG, mmol/l	0.82^{b}	1.39^{a}	0.154	0.05
TL, g/l	7.19	6.96	0.359	0.37
AST, µmol/l	5.33	5.22	0.590	0.80
ALT, µmol/l	0.06	0.07	0.013	0.50
ALP, µmol/l	56.1	61.7	17.043	0.65

CHOL: total cholesterol; HDL-C: HDL-cholesterol; LDL-C: LDL-cholesterol; TG: triglyceride; TL: total lipids; AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase; Means sharing the same superscript are not significantly different from each other (Student's unpaired T-test, $p<0.05$).

Results

Fatty acid profile and β-carotene content of feed mixtures

Fatty acid profiles of diets are presented in Table 2. The composition of fatty acid in the FP differs from the basic diets of the control. The FP contained no lauric (12:0), myristic (14:0), and vaccenic (18:1, n-7) acid. However, the basic diets contained no GLA and they had a higher content of myristic and lauric acid. The final diet of the experimental group resulted in a lower content of lauric acid, myristic acid, vaccenic acid, linoleic acid and alpha-linolenic acid when compared to the same parameters analysed in the control diet. In contrast, the content of oleic acid and GLA was higher in the feed of the experimental group. Moreover, the FP, and subsequently the diet of the experimental group, contained β-carotene which is biosynthesized by the chosen production strain.

Results of blood and immunological parameters

Results of selected biochemical parameters from the samples of broiler chicken blood are shown in Table 3. Feeding with 10% of FP from the 11th day

Table 4. Impact of fermented product on selected blood count values (n=15).

	Control	Experimental	SEM	P-value
Ec (T/l)	1.68	1.98	0.115	0.08
Ht (l/l)	0.26[a]	0.29[b]	0.006	0.01
Hb (g/l)	87.33[a]	96[b]	2.249	0.01
MCV (fl)	159.38	148.74	6.431	0.25
MCH (fmol/l)	3.34	3.8	0.141	0.20
Lc (G/l)	32	31.7	1.726	0.92
He (%)	17.4	14.6	2.67	0.47
Eo (%)	1.9[a]	1[b]	0.303	0.05
Ba (%)	0.4[a]	0.1[b]	0.160	0.05
Ly (%)	79.5	84	2.863	0.24
Mo (%)	0.3	0.3	0.152	1.00

Ec: erythrocytes; Ht: Hematocrit; Hb: hemoglobin; MCV: mean red blood cell volume; MCH: hemoglobin in the blood; Lc: leukocytes; He: heterophils; Eo: eosinophils; Ba: basophils; Ly: lymphocytes; MO: monocytes; Means sharing the same superscript are not significantly different from each other (Student's unpaired T-test, $p<0.05$).

Table 5. Effect of fermented product supplementation on selected immunological parameters (n=12).

	Control	Experimental group	SEM	P-value
CD4	25.1	21.67	2.412	0.36
CD4CD8	1.3	1.77	0.373	0.21
CD3	24.65	20.91	2.242	0.27
CD8	9.47	8.35	0.868	0.32
CD4/CD8 (IRI)	2.65	2.88	0.257	0.42
CD45	77.96	82.78	5.263	0.32
IgM	1.88[b]	3.29[a]	0.269	0.04

IRI: imunoregulatory index; Means sharing the same superscript are not significantly different from each other (Student's unpaired T-test, $p<0.05$).

had a significant effect on decreasing the total cholesterol and LDL-cholesterol and increasing the triglycerides in the experimental group ($p<0.05$). Other parameters were not affected by FP when compared to that of the control group.

Results of hematological analysis are shown in Table 4. Significantly ($p<0.05$) higher values of hematocrit and hemoglobin were detected in the experimental group. Values of eosinophils and basophils were significantly lower ($p<0.05$) in the experimental group fed with FP.

FP supplementation had a influence only on the immunological parameter IgM, which was increased in the experimental group ($p<0.05$). FP supplementation had no influence on other selected immunological parameters (Table 5). Although FP enriched with GLA and β-carotene did not affect the percentage of active phagocytes (phagocytic activity), the mean number of absorbed, fluorescently labeled *E. coli* (median fluorescence intensity – MFI) was significantly higher ($p<0.001$) in the experimental group (Fig.1).

Discussion

Microbial oils obtained by SSF represents an important source of valuable PUFAs. The advantage of the SSF process is also related to economic balance since agricultural materials are used as substrates and the obtained products can be used directly in animal feeding without subsequent processing. FP with the desired content of important substances GLA and β-carotene was prepared using SSF. We prepared FP with 3.5 % of GLA using the lower filamentous fungi *Umbelopsis isabellina* CCF2412. The amount of GLA produced depends on the fungal strain that was used and the extent to which the conditions were optimal, especially temperature during fermentation (Klempová et al. 2013). Sun et al. (2017), refer to the content of GLA ranging between 5 to 8% according to the temperature during fermentation with *Yarowia lipolytica*. The addition of prepared FP to the basic feed mixture increased the GLA and β-carotene proportion which subsequently affects the health status of the animal.

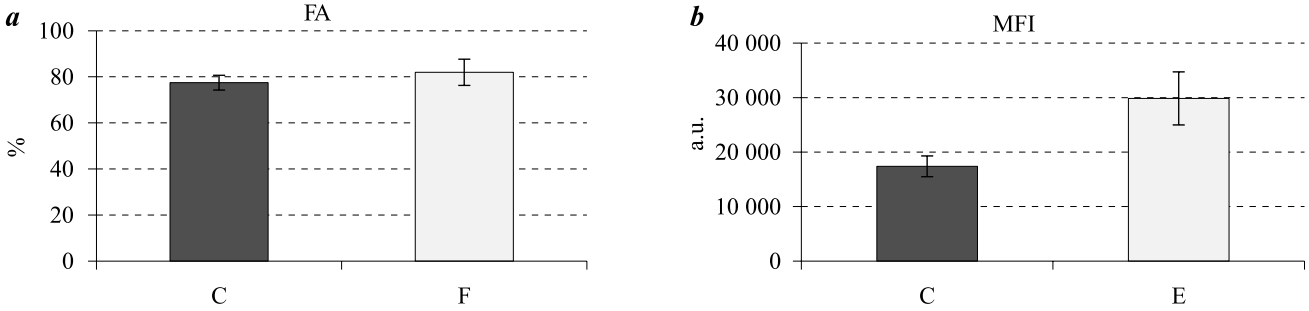

Fig. 1. Effect of fermented product application on phagocytic activity (Fig. 1a) and index of phagocytic activity expressed as mean fluorescence (MFI; Fig. 1b). IgM: percentage of selected subpopulations of lymphocytes in peripheral blood of broilers; MFI: phagocytic activity index expressed as mean fluorescence; C: control group; E: experimental group; *p<0.05.

An advantage of adding FP to a standard feed is also an increase in the production of enzymes such as phytases, amylases, proteases, lipases, etc. (data not presented).

The addition of FP to basic feed mixtures (10%) correlates to a significant decrease in cholesterol, LDL-cholesterol and conversely, an increased proportion of triacylgycerols in the blood serum of broilers. The amount of HDL cholesterol was slightly increased and the content of total lipids was lower when compared to the control. This is one of the first experiments where the impact of FP (with GLA and β-carotene) added to broiler nutrition on biochemical blood parameters was observed, thus, it is difficult to discuss our results. Ide et al. (2017) observed the effect of the following three oils: safflower oil (rich in linoleic acid), palm oil (rich in saturated acids) and oil of evening primrose origin (containing 43% GLA) on biochemical parameters in the blood serum of rats. They stated that oil with GLA, when compared with palm or safflower oils, significantly decreased the serum concentration of triacylglycerols, cholesterol and phospholipids. The concentrations of LDL fractions of cholesterol in rats given GLA oil were also less than one half of those in animals fed palm or safflower oils. These results confirm the decreasing effect of GLA on cholesterol and LDL-cholesterol. Triacylglycerol content was higher after FP feeding, in comparison to the value obtained from control. The discrepancies between the obtained results could be caused by lower GLA content, the higher total lipids and monomers of saccharides content in FP compared to feeding with pure evening primrose oil (43% GLA). These ingredients could cause the higher concentration of triacylglycerols in the blood serum as well as the increased fat storage in the body cavity (data not presented).

The decrease in HDL, LDL cholesterol and total lipids could also be caused by an increase in fiber content, especially neutral detergent fiber in the FP. It has been confirmed that feed fiber (cellulose, oat flakes) positively affects fat metabolism and subsequently the level of lipid metabolites in chicken blood serum (Safaa et al. 2014). They observed a triglyceride (TG) concentration reduction and slight total cholesterol content decrease, which resulted in a slight HDL increase and significant LDL decrease in broiler serum. Except for the triacylglycerol concentration, we observed a similar effect in our experiment. On the other hand, Najafi and Torki (2010), did not find any reaction related to the concentration of total cholesterol, triglycerides and HDL-cholesterol in the blood serum of the broilers after feeding the fermented product. Based on our results, we can state that FP helps to reduce LDL-cholesterol and the amount of total lipids but with an increase of HDL-cholesterol in broiler blood serum.

Recent studies have highlighted the role of PUFAs and their different immunological functions. PUFAs of the n-3 and n-6 series play a significant role in health by affecting modulatory molecules for inflammatory responses and also the gene expression of various molecules with bioactive potential. In the body, GLA is produced from LA as a precursor for this fatty acid by the Δ6-desaturase enzyme. GLA is then metabolized to DGLA and AA, which undergoes oxidative metabolism by lipoxygenases and cyclooxygenases to produce anti-inflammatory eicosanoids. Products of GLA metabolism also affect the expression of various genes by regulation of the gene products. These gene products play an important role in apoptosis. A study of Oliveira et al. (2010), points to the importance of GLA as a representative of n-6 PUFAs which exerts a synergic effect with n-3 PUFAs such as DHA and EPA. Concomitant administration of GLA in their study could have acted by elevating the DGLA that would also compete with AA and would favour the release of less proinflammatory eicosanoids. Studies have shown that DGLA and its subsequent metabolites are effective in the treatment of chronic inflammation, high blood pressure, inhibition of smooth muscle cell proliferation, and vasodilatation (Feng et al. 2007). The use of GLA supplement in our experiment has been suggested as a useful way

to prevent inflammatory actions in the organism. The production of GLA using non-toxigenic strains of low filamentous fungi under certain fermentation conditions could be very useful. As mentioned above, LA acts as a precursor of all n-6 fatty acids formed by subsequent desaturation and elongation. The first step in LA metabolism involves Δ6-desaturase, which allows the formation of a double bond on the sixth carbon from the carboxyl terminus. With decreased Δ6-desaturase activity, the concentration of GLA decreases. Subsequently, GLA is converted to DGLA. DGLA can be further desaturated with Δ5-desaturase to arachidonic acid. Due to limited Δ5-desaturase activity, only a small fraction of DGLA can be converted to AA. One of the possible biochemical pathways of AA is the transformation into a group of metabolites (eicosanoids), a class of paracrine hormones. Eicosanoids synthesized from AA are predominantly of inflammatory origin. Eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3), compared to GLA in fish oil as a source of n-3 PUFAs, inhibit the 5-lipoxygenase pathway and LTB_4 generation in neutrophils and monocytes in vitro. On the other hand, the effect of linolenic acid (LA, 18:2 n-6), which is the most abundantly occurring n-6 PUFA in the diet, rather tends to stimulate these responses. Evening primrose oil, one of the sources of GLA (18:3 n-6), improves atopic eczema treatment. This oil also contains a relatively high amount of LA, which increases tissue levels of prostaglandin E1 (PGEI) and suppresses chronic inflammation. It can consequently reduce LBT_4 release from polymorphonuclear neutrophils. These observations suggest a possible role of GLA in the immune regulation.

The use of fermented product with *Umbelopsis isabellinum* had a significant effect on B-lymphocytes expressed as a percentage of selected subpopulation (Fig.1). We can observe that fermented product stimulates the immune system and can confirm that GLA has immunomodulatory potential. Significant stimulation was observed only for B-lymphocytes (IgM+). These results confirm the immunomodulatory potential of fatty acids, not only in relation to nonspecific cellular immunity, but specific immune responses are also recorded. According to the results we can state that B-cell stimulation can subsequently lead to an increase in antibody production. Kwon et al. (2011), concluded that the addition of small peptides into the basal diets of piglets increased the concentration of immunoglobulin. The results of Feng et al. (2007), indicate that the fermentation process of feed could improve the performance of broilers, and increase level of IgA, IgM and total phosphorus content in serum. Guo et al. (2004), studied the effect of different PUFAs on immune function and PGE2 synthesis. They found out that PGE2 synthesis was influenced by the oil source in the broiler diet. They reached the highest level production of PGE2 in chickens fed with maize oil and the lowest levels of production were obtained from the group fed with fish oil. They explain that PGE2 was produced via the cyclooxygenase pathway, since PUFAs with less than 20 carbon atoms serve as precursors of prostaglandins and eicosanoids. We suggest that the ratio of n-3/n-6 PUFAs in the diet modulates the metabolism of prostaglandins.

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