Effect of fungal gamma-linolenic acid and beta-carotene containing prefermented feed on immunity and gut of broiler chicken

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ABSTRACT Gamma-linolenic acid (**GLA**) is a fatty acid from the ω-6 family. It is able to deliver a wide range of health benefits arising from its anti-inflammatory effects. An insufficient supply of GLA from agricultural and animal sources resulted in the development of a fermentation technique using lower filamentous fungi, which have the ability to accumulate high concentrations of GLA and beta-carotene during solid-state fermentation of cereals. The goal of this study was to observe the influence of the addition of prefermented cereal product, containing high amounts of GLA and beta-carotene, into the feed of broiler chickens on their immune status, and also the number of lactic acid bacteria and enterobacteria in gut content, which has never been studied before. Immunostimulation in the GLA group was manifested by a significant increase in the oxidative burst of phagocytes, CD4+CD8- lymphocytes in blood, and the CD4: CD8 ratio. Upregulation of gene expression for IgA in the GLA group indicates that the B-lymphocytes were stimulated at a local gut level. In the caecum, increased mRNA expression for mucin-2 and insulin-like growth factor was observed in the GLA group, which could contribute mainly to the protection of the intestinal mucosa and to better growth and regeneration of skeletal muscles. Improved immune activation and protection of the intestinal mucosa were subsequently reflected in a change of the microbial composition in gut contents; a significant reduction of enterobacteria occurred after GLA administration. We can conclude that prefermented cereals containing fungal GLA and beta-carotene represent a low-cost supplement for broiler diet having a beneficial health effect.

Key words: filamentous fungi, fermentation, gamma-linolenic acid, immune response, gut bacteria

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INTRODUCTION

In recent years, polyunsaturated fatty acids (PUFAs) have received considerable attention in both human and animal nutrition. PUFAs are important for proper body function. Western diets contain excessive levels of ω-6 PUFAs but very low levels of ω-3 PUFAs, leading to an unhealthy ω-6: ω-3 ratio of 15–30: 1, which is now very different from the original 1–2: 1 ratio of humans in the past. Such a high ω-6: ω-3 ratio is mainly caused by consumption of cereal-based food that is rich in linoleic acid (ω-6). It was confirmed that high ω-6: ω-3 ratio promotes the pathogenesis of chronic inflammatory diseases such as cardiovascular diseases, different types of cancers, obesity, diabetes or chronic bowel disease arising from proinflammatory and prothrombic activities of ω-6 PUFAs (Kang, 2005; Simopoulos, 2008; Candela et al., 2011; Khozin-Goldberg et al., 2011). In addition, cereal-based diets are deficient in some PUFAs of both ω-6 (γ-linolenic acid—GLA, dihomo-γ-linolenic acid—DGLA, and arachidonic acid—ARA) and ω-3 families (eicosapentaenoic acid—EPA and docosahexaenoic acid—DHA). Although (GLA) is a member of the ω-6 family, it is metabolized directly into DGLA and subsequently by cyclooxygenases and lipooxygenases into eicosanoids

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such as prostaglandins of series 1 (PGE1), thromboxane A1 (TXA1), and 15-hydroxyeicosatrienoic acid (15-HETrE). These products exert anti-inflammatory, anti-aggregatory, vasodilatory and cholesterol lowering actions (Kapoor and Huang, 2006). Dietary intake of GLA is negligible because it is present only in trace amounts in common foods. Main sources of GLA are seed oils from borage, blackcurrant, and evening primrose (Wu et al., 1999). Insufficient supply of GLA from agricultural and animal sources and continuing demand for more economical sources of GLA resulted in the discovery of effective biotechnological GLA production using lower filamentous fungi. Various strains of *Zygomycetes* fungi were tested for their potential to synthesize GLA as well as beta-carotene. *Umbelopsis isabellina* CCF 2412 grown in submerged culture was found to be the best strain producing both bioactive compounds in high concentrations—GLA (217 mg/L) and beta-carotene (40.7 mg/L) (Klempova et al., 2013). Moreover, this strain is also suitable for fungal solid-state fermentation. This is a useful biotechnological process for effective utilization of inexpensive waste materials and by-products from the food industry and agroindustry. These substrates are, during fungal solid-state fermentation, enriched with GLA and beta-carotene. However, the substrates and cultivation conditions must be optimized to reach a maximum yield of bioactive compounds in the product (Čertík and Klempová, 2016). Such prefermented products containing PUFAs and/or other bioactive substances can be used directly as a feed supplement for farm animals to improve their health status, increase production parameters (e.g., by reduction of anti-nutrient compounds) and modify the composition of animal products (e.g., meat or eggs) (Čertík et al., 2013; Bača et al., 2014). Although the content of GLA in food is insufficient, its supplementation through chicken meat is desirable. Moreover, knowledge of the influence of GLA on the immune system and gut microbiota in chicken is very limited; therefore, the aim of this study was to evaluate the effect of the addition of prefermented cereal product containing a high amount of GLA and beta-carotene into the feed of broiler chickens on their immune response and numbers of lactic acid bacteria and enterobacteria in gut content.

MATERIAL AND METHODS

Animals and Experimental Design

The State Veterinary and Food Administration of the Slovak Republic approved the experimental protocol number 3090/13-221 and the animals were handled and sacrificed in a humane manner. We received 100 Cobb500 male broiler chicks from Mach Hydina Budmerice s.r.o. (Hydina Slovensko s.r.o., Slovakia). The 1-d-old chicks were divided into 2 groups—control (n = 50) and experimental (n = 50). Chicks were reared in a controlled atmosphere on deep bedding with free access to water and feed. For the first 10 d, they were fed a starter diet—BR-1 (CP 221 g, Fat 38 g, Fibre 36 g, Ash 90 g, Methionine 5.6 g, Vit. A 10,800 IU, Vit. D_3 2400 IU, Vit. E 36 mg, Cu 12.3 mg, Sodium salinomycinate 70 mg; De Heus, Bučovice, Czech Republic). From day 11 to day 27, birds consumed a grower diet—BR-2 (CP 186 g, Fat 34 g, Fibre 36 g, Ash 60 g, Methionine 5.3 g, Vit. A 10,800 IU, Vit. D_3 2400 IU, Vit. E 42 mg, Cu 12 mg, Sodium salinomycinate 70 mg; De Heus, Bučovice, Czech Republic), and from day 28 to day 38, a finisher diet—BR-3 (CP 180 g, Fat 43 g, Fibre 37 g, Ash 100 g, Methionine 4.9 g, Vit. A 8100 IU, Vit. D_3 1800 IU, Vit. E 37 mg, Cu 11 mg; De Heus, Bučovice, Czech Republic). Feed mixtures BR-2 and BR-3 for the experimental chicks were supplemented with 10% of prefermented cereals containing GLA and beta-carotene. Concentrations of GLA and beta-carotene in feed mixtures are given in Table 1. On day 38, 15 birds from both groups were randomly selected for subsequent immunological and microbiological analyses. Blood samples were collected from *vena jugularis* and gastrointestinal tracts were immediately removed from the birds, which were sacrificed by cervical dislocation.

Table 1. Final concentrations of gamma-linolenic acid (GLA) and beta-catotene (mean ± standard deviation) in grower and finisher diets for control (Br-2 and Br-3) and experimental chicken (Br-2 and Br-3) supplemented with 10% of prefermented cereals (PC).

	Br-2	Br-2 + PC	Br-3	Br-3 + PC
GLA (mg/g)	0 ± 0	0.145 ± 0.004	0 ± 0	0.156 ± 0.030
Beta-carotene (μg/g)	0 ± 0	0.021 ± 0.001	0 ± 0	0.022 ± 0.001

Preparation of Prefermented Cereals

The GLA and beta-carotene-producing strain *Umbelopsis isabellina* CCF2412 (Culture Collection of Fungi, Charles University, Prague, Czech Republic) was used for the preparation of prefermented cereals. The strain was kept on potato-dextrose agar (Carl Roth, Germany) at 4°C in dark conditions. Spore suspension was prepared by cultivation of the strain on rye for 7 d (Jeennor et al., 2008). The spores were then washed with distilled water with 0.05% Tween 80, filtered and adjusted to 2.10^5 spores/ml. A total of 100 g of wheat bran (substrate) in HDPE sacks (30 × 40 cm) were moistened with 75 ml of water and soaked for 2 h at laboratory temperature. Subsequently, substrates were autoclaved for 60 min (110 kPa, 110°C). After chilling, substrates were inoculated with 20 ml of spore suspension and cultivated for 7 d at 28±1°C with additional cultivating medium (D-glucose 15 g/l, yeast extract 5 g/l) containing mycelium on the 3rd day of cultivation. Mycelium was grown in mentioned cultivating medium for 3 d at 28±1°C on rotary shaker (165 rpm; Innova 40R, New Brunswick, Canada) under constant illumination with blue light (λ_{max} = 440 nm).

Table 2. Specification of used mouse anti-chicken monoclonal antibodies.

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

The final prefermented product contained 1.2 mg/g of GLA and 0.14 μg/g of beta-carotene.

Activity of Phagocytes

Phagocytic activity was analyzed in heparinized blood with the Phagotest (ORPEGEN Pharma, Germany) and metabolic burst activity of phagocytes with the Bursttest (ORPEGEN Pharma, Germany).

Lymphocyte Isolation and Phenotypization

For the lymphocyte phenotypization, mononuclears were isolated from heparinized blood as follows: 600 μl of blood was diluted in a 1:1 ratio with PBS (MP Biomedicals, France) and carefully underlayered with 2 ml of lymphocyte separating medium LSM 1077 (PAA Laboratories GmbH, Austria). Tubes were centrifuged for 30 min at 600 × g. Lymphocytes were recovered by aspirating plasma-LSM interphase and the obtained sample was subsequently washed twice with PBS by centrifugation for 5 min at 250 × g.

For flow cytometric analysis of the lymphocyte subsets, direct staining was used, with a combination of conjugated monoclonal antibodies: CD4/CD8a/CD45 (Southern Biotech, USA). Specification of used antibodies is presented in Table 2.

Direct immunofluorescence staining was done as follows: blood was incubated for 20 min in the dark at laboratory temperature, with monoclonal antibodies. Then tubes were washed twice with 1 ml PBS by centrifugation for 5 min at 250 × g and subsequently 100 μl of PBS was added to each tube. Flow cytometric analysis was performed on a 6 color BD FACSCanto flow cytometer equipped with blue (488 nm) and red (633 nm) lasers (Becton Dickinson Biosciences, USA). Data were analyzed using the BD FACS Diva software. Position of cells was gated in FSC vs. SSC dot plot. Contaminating chicken thrombocytes were differentiated from lymphocytes based on their different side scatter profiles. Cells with the increased side-scatter correspond to thrombocytes and those with lower SSC are lymphocytes (Bertram, 1998). For the analysis, only cells with high expression of CD45 antigen were used. Proportions of lymphocytes are expressed in percentage.

Homogenization of Tissue and Isolation of Total RNA

Tissue samples from the caudal part of the caecum were cut into 20 mg pieces, placed immediately into RNA Later solution (Qiagen, UK), and stored at −70°C prior to RNA purification. Single tissue fragments were transferred into 1 ml of TRIzol Reagent (Molecular Research Center, USA), and homogenized with zirconium silica beads (BioSpec Products, USA) in a vortex mixer (Labnet, USA). To separate the phases, 50 μl of 4-bromanisole (Molecular Research Center, USA) was added. The whole content of the tube was centrifuged and the upper aqueous phase was collected for total RNA purification with the RNAeasy mini kit (Qiagen, UK), according to the manufacturer's instructions. Turbo DNA-free kit (Ambion, USA) was used for the treatment of RNA samples to remove genomic DNA. Both the purity and concentration of RNA were determined spectrophotometrically on NanoDrop 200c (Thermo Scientific, USA) and 1 μg of the total RNA immediately underwent reverse transcription with iScript cDNA Synthesis Kit (Bio-Rad, USA). The resulting cDNA was diluted 10-fold in UltraPure DNase/RNase-Free distilled water (Invitrogen, USA) and used as a template for real-time PCR, or stored at −20°C until used.

Quantitative Real-time PCR

The mRNA level of IgA, MUC-2, and IGF-2 (*insulin like growth factor 2*) was determined. In addition, mRNA relative expression of reference gene, coding GAPDH (*glyceraldehyde-3- phosphate dehydrogenase*), was used for data normalization. The primer sequences used for qPCR are listed in Table 3. All primer sets allowed DNA amplification efficiencies between 94 and 100%.

Sequence Data Collection

The IGF-2 gene sequences of chicken origin were retrieved from the 3 public databases of nucleic acids including GenBank (http://www.ncbi.nlm.nih.gov/), Silva comprehensive ribosomal RNA database (Silva, http://www.arb-silva.de/), and Ribosomal Database Project (RDP, http://rdp.cme.msu.edu/) in November and December 2016, using the following search terms: chicken, chickens, chick, chicks, poultry, broiler, hen, and hens. Sequences shorter than 150 bp were removed from the data set to avoid uncertainties in comparing and classifying short sequences that have little or no sequence overlap. Possible chimeric sequences were identified using via Chimera Slayer and UCHIME in the Mothur package (Schloss et al., 2009; Edgar et al., 2011) and were also removed. The database

Table 3. List of primers used in RT-PCR for IgA, MUC-2, and IGF-2 mRNA detection in chicks.

Primer	Sequence 5′–3′	Annealing/temperature time	References
IgA For	GTCACCGTCACCTGGACTACA	55°C/30 s	Lammers et al. (2010)
IgA Rev	ACCGATGGTCTCCTTCACATC		
Muc 2 For	GCTGATTGTCACTCACGCCTT	54°C/1 min	Smirnov et al. (2006)
Muc 2 Rev	ATCTGCCTGAATCACAGGTGC		
IGF-2 For	CTCTGCTGGAAACCTACTGT	55°C/30 s	In this study
IGF-2 Rev	GAGTACTTGGCATGAGATGG		
GAPDH For	CCTGCATCTGCCCATTT	59°C/30 s	De Boever et al. (2008)
GAPDH Rev	GGCACGCCATCACTATC		

record information associated with each of the sequences was examined, and the sequences not of poultry gut origin were removed manually.

Amplification and detection of specific products were performed via the CFX 96 RT system (Bio-Rad, USA). The cycling conditions included an initial denaturation at 94°C for 3 min, followed by 36 cycles at 93°C for 45 s. The optimal annealing temperature and time for each primer is shown in Table 3, and the final extension step was carried out for 10 min at 72°C. A melting curve ranging from 50 to 95°C, with readings taken every 0.5°C, was charted for each individual RT-PCR plate. Each sample was subjected to real-time PCR in duplicate, and mean values of duplicates were used for subsequent analysis. We also confirmed that the efficiency of amplification of each target gene (including GAPDH) was essentially 100% in the exponential phase of the reaction, where the quantification cycle (Cq) was calculated. The Cq values of the genes studied were normalized to an average Cq value of the reference gene (ΔCq), and the relative expression of each gene was calculated as $2^{-\Delta Cq}$. These expression levels were then used for comparative data analysis. Relative expression of IgA, MUC-2, and IGF-2 in the caecum was determined in 15 animals individually.

Microbiological Analysis

The bacteria-containing homogenized samples of intestinal contents obtained from the small intestine and caecum were diluted by 10-fold dilution in isotonic saline solution and plated onto MRS agar plates (Merck, Germany) to determine counts of lactic acid bacteria and onto Endo agar (HiMedia, India) for enterobacteria. After inoculation, the MRS plates were incubated in an anaerobic atmosphere using the GasPak system (Becton Dickinson, USA) for 48 h at 37°C. Endo agar plates were incubated for 24 h at 37°C aerobically. The bacterial counts are expressed in $\log_{10}$ of colony forming units per gram of content ($\log_{10}$ cfu/g).

Statistical Analysis

The data were evaluated by unpaired T-test in statistical program Graph Pad Prism version 3.00. The data are expressed as mean ± standard deviation.

RESULTS

For evaluation of the influence of prefermented feed on the immune response of broilers we tested selected parameters of non-specific as well as specific immunity. Phagocytosis as a first defence mechanism of innate cellular immunity is often affected by immunomodulators of natural origin. In our experiment, the percentage of active phagocytes (phagocytic activity) and engulfing capacity of phagocytes (expressed as mean fluorescence intensity—MFI) were not significantly influenced by addition of prefermented cereals (Figures 1a and 1b), whereas oxidative burst activity of phagocytes (expressed as index of metabolic activity—IMA) was increased in experimental animals (Figure 1c).

Proportions of CD4+ and CD8+ lymphocytes and their ratio, which is an indicator of immunomodulation, were selected for assessment of the effect on specific cellular immunity. A significantly higher proportion of CD4+CD8− lymphocytes in GLA supplemented broilers was accompanied by a significant decrease of CD4-CD8+ lymphocyte subset (Figure 2). This resulted in an increase of CD4: CD8 ratio in the experimental group (3.15 ± 0.79) in comparison to that of the control group (1.48 ± 0.43; $P < 0.001$). Double positive lymphocytes (CD4+CD8+) were not influenced.

Humoral immune answer and gut-protecting effect after application of GLA enriched feed was evaluated based on the relative expression of genes for IgA, MUC-2, and IGF-2 in the intestinal mucosa. The relative expression of IgA in the caecum was upregulated in the GLA group in comparison with the control ($P < 0.01$) (Figure 3a). The same tendency was recorded in relative expression of MUC-2 and IGF-2 in the GLA group ($P < 0.001$ and 0.01; Figures 3b and 3c).

The numbers of two representative groups of bacteria—Gram-positive beneficial lactic acid bacteria and Gram-negative enterobacteria including along with symbionts many enteropathogens were used to evaluate the affection of gut microbiota. We noted significantly lower numbers of enterobacteria in the small intestine and caecum of experimental animals in comparison with that of the animals belonging to the control group. Although numbers of lactic acid bacteria in GLA group were not affected in the small intestine, they increased in the caecum when compared to the values obtained from the control group (Table 4). Values of pH of gut

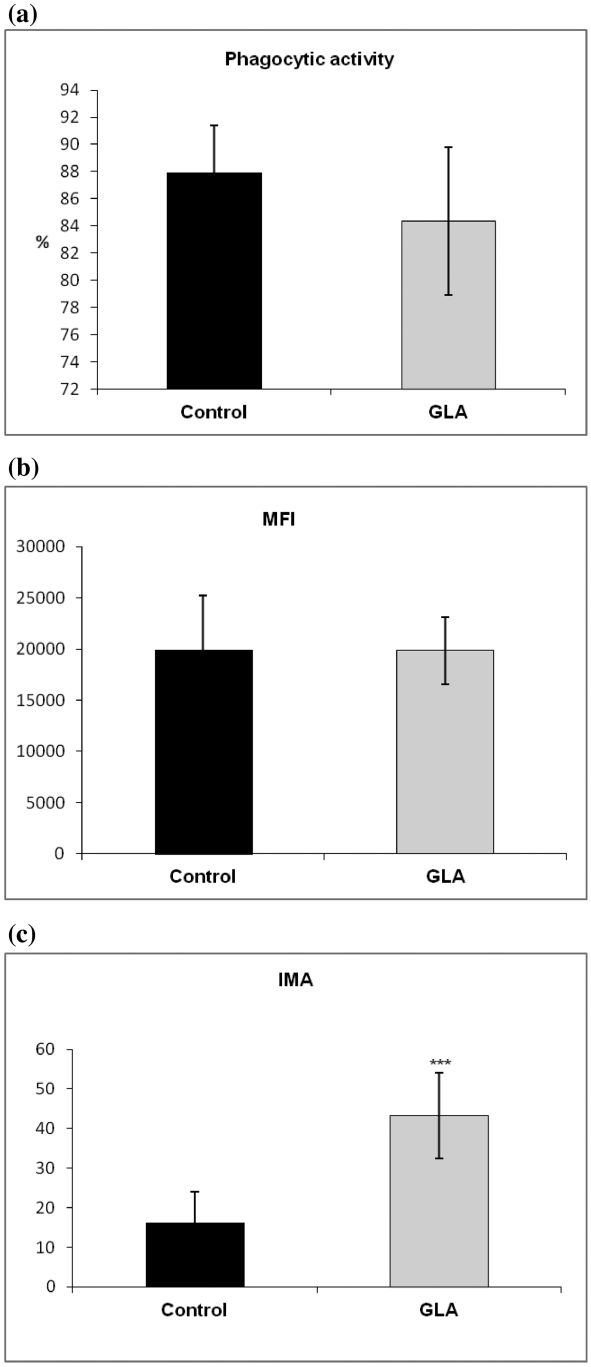

Figure 1. Influence of fungal gamma-linolenic acid (GLA) and beta-carotene containing feed on activity of phagocytes in broiler blood evaluated as: (a) percentage of active phagocytes, (b) engulfing capacity of phagocytes (expressed as mean fluorescence intensity—MFI), and (c) oxidative burst activity of phagocytes (expressed as index of metabolic activity—IMA) (n = 15). Means with different superscripts are significantly different $^{***}P < 0.001$.

contents were not influenced by application of prefermented feed.

DISCUSSION

At present, emphasis is put on the production of healthy and safe foods, especially functional foods containing ingredients with health-promoting or

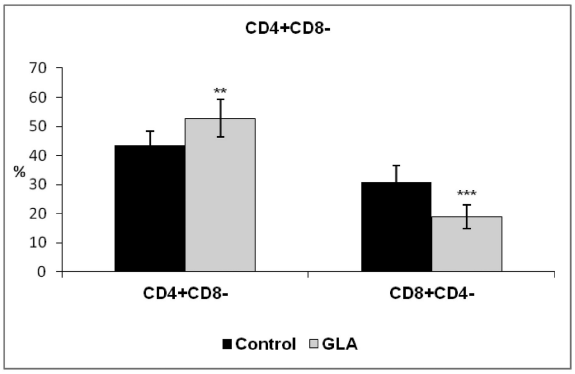

Figure 2. Percentage of CD4+CD8− (Th lymphocytes) and CD4−CD8+ (Tc lymphocytes) in blood of broiler chicken (n = 15) receiving fungal GLA and beta-carotene containing feed. Means with different superscripts are significantly different $^{**}P < 0.01$; $^{***}P < 0.001$.

disease-preventing actions. Animal products containing biologically active compounds of natural origin received by biotechnological methods represent a promising way for improvement of health of the human population. As was confirmed in previous studies, an enrichment of poultry feed with different PUFAs resulted in an increase of their concentrations in meat (Rymer and Givens, 2005; Barroeta, 2007; Bača et al., 2014) and eggs (Lewis et al., 2000; Trebunová et al., 2007; Shapira et al., 2008). Most of the authors in their experiments used different fish oils, canola, flaxseed or other plant oils as a source of PUFAs. Despite the fact that GLA is not essential for man and the essential linoleic acid from which GLA is converted in the organism is found in sufficient quantities in human food, the administration of GLA from the external sources is beneficial, because the conversion occurs with extremely low rates (Bellou et al., 2016). We developed a low-cost biotechnological method for production of GLA and beta-carotene based on optimized solid-state fermentation of cereals (e.g., wheat bran, rice bran, peeled barley, etc.) by *Zygomycetes* fungi (Čertík et al., 2013; Klempová et al., 2013). Such cereal-based bioproducts are generally recognized as safe (GRAS) for the production of food or feed supplements (Čertík and Klempová, 2016). In our previous experiment, we supplemented broiler diet with cereals prefermented by the GLA-producing fungus *Thamnidium elegans* and we noted significant improvements in the production indicators—higher final body weight and feed conversion ratio as well as fatty acid profile of breast muscles, which was manifested by a higher proportion of n-3 PUFAs, and GLA, dihomo-GLA and arachidonic acid concentrations (Bača et al., 2014).

In this experiment, we focused on the study of the immunomodulatory and gut-protecting effect of GLA and carotene containing feed for broilers where *Umbelopsis isabellina* CCF 2412 was selected as a production strain. As described above, although GLA is a member of the ω-6 family of PUFAs, it exerts anti-inflammatory activity, which is manifested by inhibition

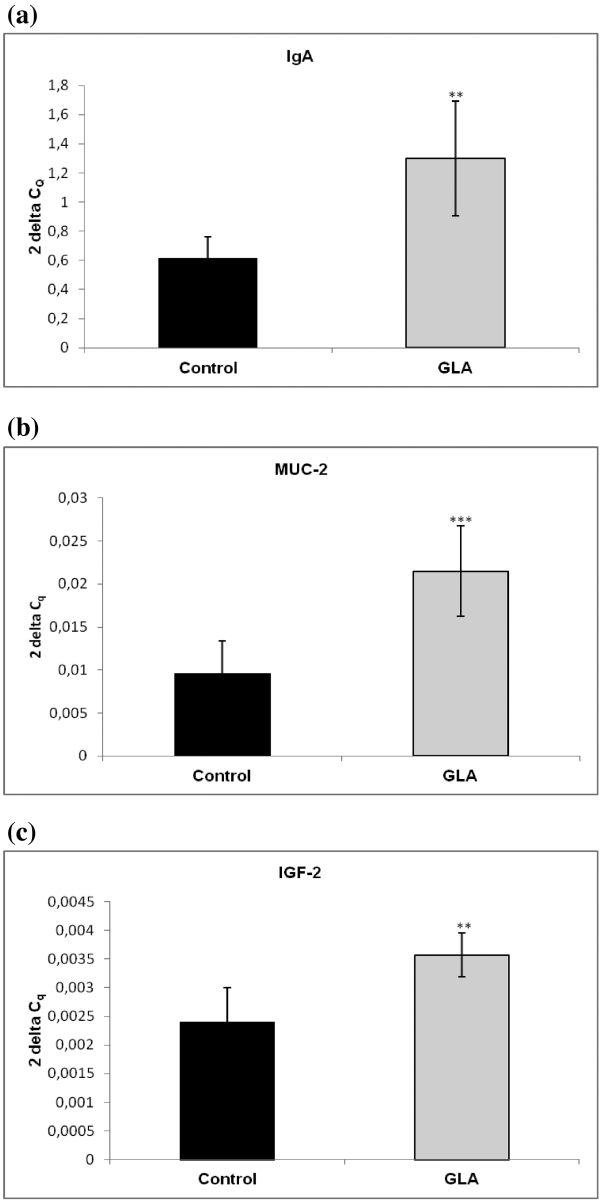

Figure 3. Relative expression level of (a) IgA, (b) MUC-2, and (c) IGF-2 in the caecum of broiler chicken (n = 15) receiving fungal gamma-linolenic acid (GLA) and beta-carotene containing feed. Results at each time point are the median of $2^{-\Delta Cq}$. Means with different superscripts are significantly different $^{**}P < 0.01$; $^{***}P < 0.001$.

of IL-2 and IFN-γ production by lymphocytes, inhibition of lymphocyte proliferation, or inhibition of IL-1, IL-6 and TNF production by macrophages (Calder et al., 2002). PUFAs are incorporated in cell membranes of immune cells thereby they influence membrane fluidity, cellular signal transduction, and thus cell proliferation, adhesion and survival (Hancock, 2006). They also have a direct effect on the activity of phagocytes. Although the results regarding the effect of the fatty acids on phagocytic activity are sometimes contradictory, most studies have confirmed inhibitory effect of ω-3 and stimulatory effect of ω-6 PUFAs on neutrophil recruitment, migration and engulfing activity (Rodrigues et al., 2016). In our study, supplementation of GLA did not influence phagocytic (engulfing) activity. This can be explained by the fact that GLA does not accumulate in cell membranes, but concentration of its derivate DGLA increases dose-dependently when GLA is supplemented in feed. Moreover, the activity of desaturase, which converts DGLA to arachidonic acid, is relatively low in immune cells (Johnson et al., 1997). On the other hand, more authors reported stimulatory effect of different PUFAs on production of reactive oxygen species by neutrophils (Hatanaka et al., 2006; Paschoal et al., 2013; Rodrigues et al., 2016) and we observed significantly higher oxidative burst activity of phagocytes in chickens receiving prefermented feed, in comparison with the control, which facilitates phagocytes killing capacity. Interestingly, we noted significant increase of CD4+CD8- lymphocyte subset as well as CD4: CD8 ratio in GLA supplemented animals in comparison to that of the control group, indicating immune stimulation. Because there are no such studies on poultry, it was not possible to compare our results with other authors. Kaku et al. (2001) found out that dietary GLA promoted immunoglobulin (IgA, IgG and IgM) production by rat mesenteric lymph node lymphocytes. In agreement with this finding, relative expression of IgA in the caecum of broilers receiving GLA rich feed was upregulated in comparison with poultry belonging to the control group. Insulin-like growth factors promote both muscle cell proliferation and differentiation and play a key role in muscle regeneration and hypertrophy (Zhou et al., 2015). Increased mRNA expression for insulin-like growth factor in GLA group can contribute to better growth and regeneration of skeletal muscles in broilers. This was confirmed by a higher final body weight and better feed conversion ratio (unpublished data). MUC-2 mucin is a major component of the intestinal mucus protecting the epithelium from bacteria by promoting their clearance and separating them from the epithelial cells, thereby inhibiting

Table 4. Numbers of enterobacteria and lactic acid bacteria in the small intestine and caecum of broilers (n = 15) expressed as $\log_{10}$ cfu/g of gut content and pH values (mean ± standard deviation).

	Enterobacteriaceae		Lactic acid bacteria		pH	
	Small intestine	Caecum	Small intestine	Caecum	Small intestine	Caecum
Control	5.6 ± 0.70	8.1 ± 1.00	8.7 ± 0.44	8.6 ± 0.52	5.0 ± 0.48	6.6 ± 0.59
GLA	2.8 ± 2.53	4.0 ± 3.07	8.6 ± 0.40	9.1 ± 0.52	5.0 ± 0.39	6.7 ± 0.65
T-test	$P < 0.01$	$P < 0.01$	ns	$P < 0.01$	ns	ns

ns—non-significant ($P > 0.05$).

inflammation and infection (Hansson, 2012). Increased relative expression of MUC-2 in caecum of GLA supplemented broilers was accompanied by significantly lower counts of enterobacteria and higher counts of lactic acid bacteria in caecum, and evidentially, this confers higher protection against intestinal pathogens. The fact that PUFAs can affect the growth of bacteria has been known for a long time. Already at the beginning of the 20th century, scientists have observed the potent antibacterial effect of long-chain unsaturated fatty acids on pathogenic bacteria such as pneumococci, haemolytic streptococci, meningococci or *Mycobacterium tuberculosis* (Pollock, 1949). At the same time, the influence on beneficial lactic acid bacteria was also studied and the authors received contradictory results. Bauernfeind et al. (1942) reported that certain unsaturated fatty acids stimulated the growth of *Lactobacillus helveticus* whereas Kodicek and Worden (1945) noted that oleic, linoleic and linolenic acids inhibited the growth and acid production of *L. helveticus* and this effect was concentration dependent. Both results were later confirmed. Kankaanpää et al. (2001) found out that the effect of PUFAs on lactic acid bacteria is dependent on the type of PUFA, its concentration and strain of bacteria. Concerning GLA, low concentrations (at 5 μl/ml) promoted the growth of *L. casei* Shirota and *Lactobacillus* GG, but showed no influence on *L. bulgaricus*, whereas higher concentrations ($>$ 10 μl/ml) suppressed the growth as well as mucus adhesion of all tree strains. However, all mentioned findings were obtained in in vitro experiments and they do not have to correspond with the effect of PUFAs in the organism. In our previous experiments on pigs, we have noted stimulatory effect of omega-3 PUFAs on lactobacilli adhesion to the intestinal mucosa accompanied by inhibiting the digestive tract pathogens (Bomba et al., 2002; Nemcova et al., 2012). Similar results received Ringo et al. (1998) in fish. However, similarly focused in vivo experiments with GLA have not yet been performed. As the adhesion to intestinal mucosal surfaces is critical for colonization and immunological action of bacteria, these results indicate that health promoting effect of beneficial microbiota can be modulated by dietary PUFAs.

In conclusion, prefermented cereals containing fungal GLA and carotene represent a low-cost supplement for broiler diet having immunomodulatory (by stimulating cellular immune response and gut IgA expression) and gut-protecting (by increasing expression levels for mucin 2 and insulin like growth factor 2) effects on the chicken. Received results showed that the used concentration was optimal for achieving a positive influence on the gut microbiota and immunity of broilers.

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