

SUMMARY

The PhD thesis entitled *Effect of natural antioxidants on lymphoid tissue associated with the digestive tract in free-range birds* aimed to evaluate the nutraceutical effect of curcumin and ZooBior fed to free-range broiler chicks.

The paper was written and structured in two main parts according to current scientific standards. A total of 426 bibliographic sources were used for documentation.

The first part, structured in four chapters, reflects the synthesis of the literature on intestinal morphophysiology in birds, gut-associated lymphoid tissue in birds, mucosal homeostasis and barrier function, transcellular or paracellular transport, natural antioxidants used as nutritional supplements in poultry feed. Data on *Curcuma longa* and *Spirulina platensis* have been synthesised.

Part 2 *Own research*, is structured in 6 chapters.

It starts with a description of the organisational framework, followed by a description of the aim, the objectives followed to achieve it, and ends with a presentation of the final conclusions on the studies addressed. Each chapter describes the objectives of the studies, the materials and methods used, the results obtained, their discussion and the outlining of partial conclusions.

With the banning of antibiotics in the European Union in 2006, alternatives were sought to improve animal health. Some of the most suitable, according to studies, are phytogetic additives added to poultry diets. Intestinal inflammation in chickens affects function, gut integrity and growth performance. Dietary strategies to inhibit the intestinal inflammatory process are the main targets in this regard.

Numerous animal studies have shown that dietary polyphenols, particularly representatives of the flavonoid group, are able to attenuate inflammation by modulating NF- κ B and Nrf2 activity. However, their potential anti-inflammatory effects have been investigated relatively little to date in broiler chickens.

It should be noted that digestive mucosa-associated lymphoid tissue (DLAMT) is a first line of defence against harmful antigens that enter the body during feeding and depending on location, is present as aggregates of lymphoid cells and also as cells organised into lymphoid follicles and tonsils (Casteleyn et al, 2010). Mucosa-associated lymphoid tissues are highly developed in chickens in numerous locations and especially in the gastrointestinal tract and include structures unique to birds, such as Meckel's diverticulum and cecal tonsils. The TLAMD system is characterised by the production of IgA and comprises over 2/3 of all lymphoid cells of the entire immune system. Approximately 80% of all immunoglobulins and 50% of lymphocytes are produced in the gut. Lymphoid tissue is composed of an interfollicular space in which T-cells accumulate and B-cells form lymphoid cells; all of which are covered by specialized follicle-associated epithelium (FAE). It can be argued that the finely tuned defence system of the intestinal barrier is influenced by a variety of factors and is the result of an interaction between the mucosa, the microbiota and the intestinal immune system (Hoffmann et al, 2009). The intestinal epithelium is also protected via the mucus layer from commensal microorganisms normally present in the intestinal lumen, as well as from pathogenic bacteria or viruses. The mucus provides protection not only through a physical barrier, but also through antimicrobial peptides secreted by the epithelium into the mucus layer and bound within it.

Among the phytogetic additives used in the bird diet we evaluated Turmeric and the ZooBior product based on *Spirulina*.

Turmeric (*Curcuma longa*) contains an active component called curcumin (WuthiUdomler et al., 2000 and Mashhadani, 2015) ranging from 2 to 5% in turmeric. Turmeric (turmeric root) is commonly used as a feed additive as it has intestinal inflammation relieving activity, is potent antiviral, antibacterial, antifungal and and is successfully used in intensive poultry production and beyond (Choudhury et al, 2019).

Blue-green algae (*Spirulina platensis*), contains a high proportion of protein (50-70% in dry weight) being considered a good source of essential amino acids, a point confirmed by Farag M. R. et al. Ross and Dominy (1990) analysed the composition of *Spirulina platensis* algae and found similar values of crude protein (60.5%) and phosphorus (0.95%), a similar amount of dry matter (94.5%). The *Spirulina*-based ZooBior product improves the health of birds and mammals and further increases production efficiency by modulating the microbiota and improving the function of lymphoid tissue associated with the intestinal mucosa.

The clinical research was carried out in a household system and the histological, immunohistochemical and histomorphometric study was performed in the Faculty of Veterinary Medicine of the "Ion Ionescu de la Brad" University of Life Sciences in Iasi, in the Victoria Zinca Histology and Embryology Laboratory of the Faculty of Veterinary Medicine. The research consists of two stages: clinical study carried out in a household system and histological, immunohistochemical and histomorphometric evaluation of the intestine. Broiler chicks were exposed to turmeric powder and young laying hens taken in the study to the nutritional supplement - ZooBioR2 (ZBR2).

Turmeric was administered one day after hatching for 42 days to broiler chicks. The turmeric powder (brand: HerbalSana; country of origin: India) used in the experiment had the following nutritional value as stated on the label: total fat - 9.7 g, of which 1.8 g saturated fatty acids; carbohydrates - 44.4 g, of which 3.2 g sugars; dietary fibre - 22.7 g; protein - 9.7 g; salt - 0.07 g/100 g turmeric powder. Curcumin and total phenol content were determined from the turmeric powder. The curcumin content was 1.13 ± 0.06 g / 100 g turmeric powder, determined spectrophotometrically according to literature (Chauhan et al, 1999). The total phenol content determined was 2.703 ± 0.110 g gallic acid equivalents (GAE) / 100 g turmeric powder, as determined according to a previously described method (Luca et al, 2022).

Birds in the control group (C) were fed a commercial corn-soybean diet: starter (1-14 days), broiler (15-28 days), finisher (29 - 42 days) according to NRC (1994) guidelines. The birds received additional turmeric powder/kg feed as follows: 5 g/kg (LE1), 10 g/kg (E2). 20 g/kg (E3).

The addition of curcumin to the diet of the chicks increased the height of the duodenal villi in the E2 group and decreased it in the control group. Among the experimental groups, the lowest value of villus height was observed in group E3. The highest crypt depth values are found in batch E3 (2% curcumin). The lowest values are in batch E2 (1% curcumin). Batch 1 (0.5%) has values close to those of the control batch. The best ratio villous height: crypt depth is observed in batch E2. The worst ratio of all the plots is observed in plot E3. The diameter of the villi at the base of the villi is the largest in lot E3 and the smallest in lot E1.

Integrity of the intestinal epithelium, was very well preserved in LE1 chicks and lower in the other flocks, the lowest being in the control flock. Diffuse lymphoid infiltrates associated with intestinal epithelial detachment were more intense in the control and E3 groups and reduced in the E1 and E2 groups.

In terms of inflammatory reactivity, in this experiment, a positive expression for IL-6 was observed in the control and E3 groups, suggesting the existence of an inflammatory process and very reduced in LE1 and LE2. Turmeric powder reduced inflammation in the intestine of chickens in groups E1 and E2. TNF alpha expression is more intense in the control groups, moderate in group E3 and reduced in LE1 and LE2. TNFalpha expression correlates with IL-6 and attests to the presence of an inflammatory process in the control group which is attenuated in the other groups, the lowest being in LE2. Pax-7 expression is positive in all groups. The expression of the p65 gene was also evaluated and shows a similar expression to IL6 and TNF alpha, thus attesting to the inflammatory process that is reduced by turmeric supplementation.

Supplementation with low and medium doses of turmeric leads to increased expression of Nrf2, a marker that is activated by these supplements. Nrf2 reaches the nucleus and determines the transcription of the antioxidant genes superoxide dismutase and catalase, thus reducing oxidative stress during inflammation.

The addition of 1% curcumin to the diet of broiler chickens in batch 2 resulted in overexpression of Nrf2. Addition of 1% and 2% curcumin to the diet of broiler chickens from E2 and E3 respectively resulted in a marked decrease in p65 expression in the cecum. Addition of 1% curcumin to the diet of E2 birds resulted in the most significant reduction in p65 expression and the most marked increase in Nrf2 expression in the cecum. There is a link in oxido-reduction processes between increased Nrf2 expression and decreased p65 expression in the gut.

The lymphoid tissue of the cecal tonsils shows large lymphoepithelial agglomerations, predominantly formed by LT in the control group. In the other batches, lymph nodes also appear in which differentiation of B lymphocytes into IgA-secreting plasma cells occurs. Their number is variable, somewhat close numerically to moderate size. We note the presence of epithelial berirelia intact in LE2 and slightly destroyed depending on the batch. In the control batch, oedema and detachment of the columnar absorptive epithelium was reported. Taking into account morphological peculiarities, lymphoid tissue reactivity, low feed conversion ratio, lowest feed intake and weight of the chicks, we conclude that at the end of the rearing period the addition of 1% curcumin in the diet led to the best results and in the first 14 days of life the addition of 0.5% curcumin in the feed led to significantly better results.

Regarding feed consumption, the lowest amount consumed was obtained by adding 0.5% curcumin in the diet of the chicks in the first growing period with values of 110g in the first 7 days, 390g between 7 and 14 days and 900g until day 21. Between day 22 and 42 the lowest amount of feed consumed was obtained in the E2 batch with values of 1400g until day 29 and 2000g until day 42. The lowest feed conversion ratio was observed in batch 2 by adding 1% curcumin to the diet of the chicks.

The best chick weights were observed in batch E1 (0.5% curcumin) but batch E2 (1% curcumin) had very similar values and the differences were not significant. The weight of the chicks in batch E1 on day 42 was 2989.80±32.30 g. Economically it is most advantageous to add 1% curcumin to the diet to increase body weight, as the feed conversion ratio and feed consumption values are the lowest among the experimental groups.

The addition of 2% curcumin to the feed at the end of the experimental period, has negative effects on the above mentioned indicators. The other indicators have similar values to the control batch, so the addition of 2% curcumin is not economically justified.

ZBR2 is a complex natural remedy containing biologically active compounds derived from the cyanobacterium *Spirulina (Arthrospira) platensis* (SP). It contains: amino acids, including free immunoreactive ones and as component parts of peptides and proteins, respectively; polysaccharides; sulphated polysaccharides; phospholipids and trace elements zinc and selenium (Rotaru, 2016, Rudic et al, 2004).

The research was conducted for 60 days on 70 23-week-old laying hens of the Braun-Nic hybrid, divided into 5 flocks, each with 14 birds. The diets consisted of 5 mg/kg feed (E1); 10 mg/kg feed (E2); 15 mg/kg feed (E3); 20 mg/kg Zoobior feed (E4).

Morphometric parameters characterizing mucosal architecture varied between the control and experimental groups to which ZBR2 was added. Statistically significant differences were found in the length of the villi and the depth of the crypts in the duodenum, jejunum, ileum and cecum between the experimental and control groups.

The highest height of intestinal villi was observed at E 1 irrespective of the segment studied and the lowest values of villi height could be identified at E4, with the addition of the highest concentration of spirulina. Muscle thickness had the lowest values in group 4 and the highest values in group E1. The thickness of the muscle layer of the duodenum and caecum was significantly lower in the experimental groups compared to the control group, and was also associated with better feed digestibility.

The diameter of the villi at their base was the largest in batch 1 and the smallest in batch 4. The values were significantly lower in the experimental batches than in the control batch. In our study *S. platensis*

had no significant effect on the height of the villi and the depth of the duodenal crypts. Inclusion of the ZBR2 compound in the ration resulted in a slight decrease in intestinal villus height and crypt depth in all intestinal segments. A decrease in crypt depth is an indicator of a higher rate of epithelial tissue turnover and could be implicated in better feed digestibility.

Expression of IL-6, TNF alpha and p65 was more intense in the control group and lower from E4, E3, E2 to E1. Being markers of inflammation, the presence of inflammation in the control group was found to decrease progressively and was reduced in LE1, denoting the anti-inflammatory role of ZBR2. Nrf2 expression was very intense in LE1 and progressively reduced from E2, E3, E4 in the control group. This difference in Nrf2 expression explains the antioxidant and anti-inflammatory effect of ZBR2. Lymphoepithelial clusters, with reduced epithelial barrier integrity, oedema, was found in the control and LE4 group. In all the experimental groups, the number and size of lymph nodes in these agglomerations were variable. In LE1, small lymph nodes were observed, in the other groups E2, E3 and E4 they were of variable size, slightly larger than in LE1. We consider that the presence of these lymph nodes is related to the production of IgA secreting plasma cells present in the intestine which increases the local immune response,

The best weight of the chicks was found in E1; the E2 batch had very similar values, the differences not being statistically significant. Addition of ZBR2 compound to the diet improved feed conversion ratio and weight of the chicks. The abundant essential amino acids found in spirulina play a crucial role in improving general health and body weight while alleviating health disorders and mitigating the effects of oxidative stress.

Conflicting findings between studies on the effects of spirulina supplementation in broiler diets can be attributed to various factors: spirulina dose, feed preparation, age of chickens, hybrids, housing conditions. Spirulina also has a beneficial impact on the gastrointestinal flora, promoting a healthier balance of micro-organisms and also enhances the activities of digestive enzymes, leading to improved overall digestion of dry matter and nitrogen (Park et al, 2018).

Clinically and economically, a lower dose of spirulina in the diet is recommended for body weight gain as feed conversion ratio values and feed consumption are lowest in experimental groups. The higher dose of spirulina resulted in a significant reduction in villi height, villi height: crypt depth ratio and bird weight.

The anti-inflammatory properties of spirulina can be attributed to its ability to inhibit the synthesis of pro-inflammatory cytokines such as TNF-alpha, IL-6 and p65, as well as increasing Nrf2 activity.

Administration of phenolic compounds from curcumin and spirulina reduced intestinal inflammation, improved nutrient digestibility and metabolism in birds.