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INVESTIGATION OF PHYSIOLOGICAL AND ANTIMICROBIAL EFFECTS OF MILK THISTLE IN FATTENING DUCKS

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1. Background and objectives

The production of food products from waterfowl in Hungary is an export-oriented sector, where closed housing system and intensive feeding are spreading more and more due to avian influenza and the increase in production efficiency. In addition to public health expectations, there is also a growing consumer demand for products from flocks raised without the use of antibiotics. A successful reduction of antibiotic use can only be achieved through the uniform and coordinated development of farm animal health, housing technology and feeding management. In order to replace antibiotics, in recent decades, more and more studies have been conducted with a wide variety of feed supplements, including phytobiotics based on the medicinal effects of plants. When choosing the medicinal plant to be investigated in our experiments, it was an important aspect to use a species that can be easily and cost-effectively grown and processed in Hungary. We chose milk thistle (*Silybum marianum*), whose proven success in medical applications is due to the antitoxin, antioxidant, protein synthesis-enhancing, antifibrotic, antitumor, antiviral and anti-inflammatory effects of the plant's active ingredients at the cellular level. Through its beneficial antimicrobial effects in the intestine, it can contribute to the development of a healthy intestinal flora. All of these effects can also apply during duck fattening and liver production. Since there is still quite few of research and literature data available regarding the use of milk thistle in feeding ducks, I would like to contribute to the results related to this versatile plant species with my investigations.

During my doctoral research, I set the following objectives in connection with the *in vitro* and *in vivo* investigation of the effects of several milk thistle products (milk thistle seed, milk thistle seed cake, milk thistle seed oil):

During my first experiment, our aim was to investigate the antimicrobial effect of milk thistle seed cake extract and milk thistle seed oil on the growth of four groups of microbes from the ileal contents of ducks using the agar culture method. During the *in vitro* experiment, I wanted to investigate the bacterial growth rates of milk

thistle preparations at concentrations of 0.5 and 1.5 g/100 ml of culture medium for *coliform* (all and faecal) and *Enterococcus* facultative pathogenic strains of the *Enterobacteriaceae* family, as well as *Lactobacillus* strains.

The objective of my second experiment was to investigate the effects of dietary supplementation of milk thistle seed (0.5%), seed cake (0.5%) and seed oil (0.1%) in ducks fed diets naturally contaminated with deoxynivalenol (DON) and zearalenone (ZEN) mycotoxins. The measured parameters were the body weight of ducks, the relative weight and the histopathological characteristics of certain organs (liver, spleen, bursa of Fabricius), and the clinical-chemical parameters of the blood serum (AST and ALT activity, glucose, cholesterol, triglyceride, creatinine and uric acid concentration).

In my third experiment, the dietary addition of 2% milk thistle seed oil with or without a symbiotic supplement containing fructooligosaccharides and three types of bacteria strains (*Bifidobacterium*, *Lactobacillus*, *Enterococcus*) and a control diet supplemented with 2% sunflower oil were investigated during the fattening of ducks. In this experiment, my aim was to determine the performance (body weight, weight gain, feed intake, feed conversion ratio) and certain carcass parameters (relative weight of breast, thighs and liver), the activity of pancreatic enzymes (α-amylase, lipase, trypsin), measurement of the short-chain fatty acid concentration of the caecal contents, the apparent ileal digestibility of amino acids, the most important parameters characterizing the functioning of the liver antioxidant system (GSH, GSHPx, MDA).

2. Materials and methods

In accordance with the described research objectives, three different experiments were performed – one *in vitro* and two *in vivo*. The methodological details of the experiments are presented below.

2.1. In vitro determination of the antimicrobial effect of milk thistle seed cake extract and milk thistle seed oil

2.1.1. Experimental animals

My studies were conducted at the Institute of Physiology and Nutrition of the Hungarian University of Agricultural and Life Sciences (former National Center for Agricultural Research and Innovation – Institute of Animal Breeding, Nutrition and Meat Research, Herceghalom). A total of eight eight-week-old Hungarian white ducks were used in the experiment which arrived the day before slaughter from the duck breeder Tibor Miskolczi (2086 Tinnye, Damjanich u. 63). The animals had not previously been treated with drugs, vaccines, nor had they received an alternative growth promoter supplement. The basic principles of organic animal husbandry were applied at the farm where the ducks were raised. Before slaughter, the ducks consumed feed and drinking water *ad libitum*, which also provided the largest possible amount of intestinal content necessary for sampling.

2.1.2. Sampling

During the slaughter, the animals were properly fixed and bled through the jugular vein. The bodies of experimental animals did not receive any heat or other treatments. When dissecting fresh carcasses, after cutting the skin from the neck to the cloaca, the sternum was removed by cutting through the ribs and the abdominal cavity was opened. After the liver was removed, the intestines became visible. In order to remove the intestines intact, the upper digestive tract was ligated directly proximal to the stomach, while the lower ligation took place in the section of the large intestine

close to the cloaca. After removing the intestines intact, I spread the removed section on a tray, then cut the intestines at the entry of the caecum. The ileal sample to be examined was obtained under sterile conditions from the ileum section 30-40 cm proximal to the cut (5 g/duck on average). The individual samples were pooled and homogenized, then stored frozen at -20 °C until the analyses.

2.1.3. Microbiological investigations and treatments

The antibacterial effect of two forms of milk thistle (seed cake and oil) on four groups of intestinal flora were investigated. The milk thistle seed cake and oil were bought from Safimpex Ltd (2600 Vác, Pipitér utca 6.). The active ingredients of the milk thistle seed cake were extracted by soaking in distilled water for two hours at 37°C, while the plant oil was made soluble with an emulsifier (hexane).

The experimental groups were as follows:

1st group: control

2nd group: milk thistle seed cake extract, 0.5 g/100 ml culture media

3rd group: milk thistle seed cake extract, 1.5 g/100 ml culture media

4th group: milk thistle seed oil (emulsified), 0.5 g/100 ml culture media

5th group: milk thistle seed oil (emulsified), 1.5 g/100 ml culture media

During my investigation based on the classical agar culture method the following experimental media were used: MRS (de Man, Rogosa, Sharpe; Scharlab Hungary) for the *Lactobacillus* strains, SB (Slanetz and Bartley; Scharlab Hungary) for the *Enterococcus* strains while the *coliform* strains belonging to the *Enterobacteriaceae* family were cultured on MacConkey (Scharlab Hungary) type selective media. After sterilization, the milk thistle-based substances were added to the culture media according to the experimental groups. Samples of 1 g of intestinal contents were weighed and added to 90 ml of Ringer's solution in an Erlenmeyer flask, then a dilution series until 10^5 were made. The appropriate dilutions (10^1, 10^3, 10^4, 10^5) in a quantity of 1 ml each were pipetted into a Petri dish, 15 ml of the appropriate medium

was added, cooled to 50 °C, and mixed gently. The samples were incubated according to the regulations at a constant temperature of 37°C (*Lactobacillus, Enterococcus*, all *coliforms*) and 44°C (faecal *coliforms*) for 48 hours. After the incubation period, the Petri dishes were evaluated and information was obtained about the colony-forming unit (CFU) of total and faecal *coliform, Enterococcus* and *Lactobacillus* bacteria in the intestinal samples and the minimum inhibitory concentration (MIC) of treatments on intestinal bacteria. For each experimental group, the results of three measurement series (n=3) were averaged. The logCFU value measured for each treatment was compared to the logCFU value of the control treatment (100%) and thus the inhibition % was determined.

2.1.4. Statistical evaluation

In the case of the logCFU averages obtained as a result of agar cultivation, Kruskal-Wallis analysis was used to evaluate the effects of the experimental treatments, and after a significant effect was detected, the Mann-Whitney test was used to examine the differences between the groups. The significance level was et at $P \leq 0.05$. The statistical analysis was performed with the SPSS 20.0 for Windows software package.

2.2. The effect of various milk thistle supplements of diets contaminated with mycotoxins on the clinical chemistry of blood serum and histopathological characteristics of some organs

2.2.1. Experimental Animals and Dietary Treatments

The experiment was conducted at the Institute of Physiology and Nutrition of the Hungarian University of Agricultural and Life Sciences (former National Center for Agricultural Research and Innovation – Institute of Animal Breeding, Nutrition and Meat Research, Herceghalom). A total of 80, one-day-old female White Hungarian ducks were purchased from the duck breeder Tibor Miskolczi (2086 Tinnye, Dam-

janich u. 63), housed in deep litter floor pens and randomly divided into four treatment groups with five pens per treatment (n = 20/treatment, n = 4/pen). The animals did not receive any pharmacological treatments or vaccines during the experiment. Starter and grower diets were fed from day 0 to 14 and from day 15 to 42, respectively. The dietary treatments consisted of the control diet (A), the control diet supplemented on top with 0.1% milk thistle seed oil (B), 0.5% milk thistle seed cake (C), or 0.5% milk thistle seed (D). Milk thistle supplements were purchased from Safimpex Ltd. (2600 Vác, Pipitér utca 6.). The corn used for the experimental diets was naturally contaminated with mycotoxins. The analysed concentrations of mycotoxins in corn were 4.9 mg/kg deoxynivalenol (DON) and 0.66 mg/kg zearalenon (ZEN). The calculated concentration of DON was 3.43 and 3.72 mg/kg, while the calculated concentration of ZEN was 0.46 and 0.50 mg/kg in the starter and grower diets, respectively. The calculated dietary concentrations of DON in the experimental diets were lower than the maximum guideline value for complete poultry diets (5.0 mg/kg; Directive 2006/576/EC). However, the calculated concentration of ZEN almost exceeded the guideline value in the starter diet (0.5 mg/kg; 2006/576/EC), or it even reached the reference value in the grower diet. The concentration of fusariotoxins other than DON and ZEN were not measured in the experimental diets, but their presence cannot be ruled out. Feed and water were provided ad libitum throughout the study. Chopped straw was used as bedding material. Room temperature, humidity, and ventilation rate were controlled according to the needs of the duck breed during the experiment. In order to ensure an optimal temperature for the young ducks, an infrared lamp was used in each pen. On placement day, the average temperature under the infrared lamps was 32-33°C, while in the room it was 30°C, which was gradually reduced according to age requirements. From the 22nd day until the end of the experiment, a constant temperature of 18°C was set measured at the level of the animals. The experiment complied with the Government Decree 40/2013. (II. 14.) on animal experiments and approved by the Animal Welfare Committee of the MATE Georgikon Campus (MÁB-1/2017).

2.2.2. Clinical Status, Measurement of Organ Weights, and Sampling

The clinical status of the ducks was observed daily and the signs of feed refusal, diarrhea, atypical behaviour of birds (tonic immobility reaction or strutting behaviour) and Vet. Sci. 2023, 10, 100 4 of 13 the presence of undigested feed in excreta were examined. On days 14, 28, and 42 five (d14 and 28) and eight (d42) ducks per treatment group were randomly selected, their individual body weight was measured, and blood samples of 1 mL per animal were collected from the wing vein (*v. cutanea ulnaris*). Serum was separated after centrifugation at 5500×g for 10 min, aspirated by pipette and transferred into 1.5 mL Eppendorf tubes and stored at −20 ◦C until further analysis. Selected ducks were terminally anaesthetized with carbon dioxide and the liver, spleen, and bursa of Fabricius were removed and weighed. The oropharyngeal cavity and oesophagus of birds were inspected macroscopically. Relative organ weights were calculated as a percentage of the live body weight of animals measured before slaughter. For histological examination, samples of 10 g of the listed organs were fixed in a 10% buffered formaldehyde solution.

2.2.3. Analytical and Histopathological Examination Methods

Concentrations of DON and ZEN in corn were determined using the commercially available ELISA kits RIDASCREEN™ DON and RIDASCREEN™ ZEN (R-Biopharm GmbH, Darmstadt, Germany). The clinical chemical analyses of serum samples (AST and ALT activity, concentrations of glucose, cholesterol, triglyceride, creatinine, and uric acid) were performed by Vet-Med-Labor Ltd. using colorimetric assay kits (Diagnosticum Co., Budapest, Hungary) based on spectrophotometric methods. Histopathological examinations were performed by Autopsy KKT (Budapest, Hungary). The liver, spleen, and bursa of Fabricius samples in formaldehyde solution were embedded in paraffin and 5 μm thick sections were stained with haematoxylin and eosin. Tissue morphology was observed under a light microscope. The mean histological score was derived from the grade and stage of histological

lesions seen in the investigated organs of the affected animals. The listed lesions were characterized per animal (1 point = mild, 2 points = medium, 3 points = high-grade alterations) and then mean score values were calculated in the group. The extent of vacuolar degeneration of hepatocytes, solitary hepatocyte necrosis, individual cell deaths of the mononuclear phagocyte system (MPS), focal lymphocytic and histiocytic interstitial infiltrates and interstitial fibrosis in liver samples, as well as lymphocyte counts in spleen and bursa of Fabricius samples, were evaluated

2.2.4. Statistical analyses

Data of organ weights and serum clinical chemical parameters were evaluated by one-way analysis of variance (ANOVA) after testing of normal distribution (Kolmogorov–Smirnov test) of data and homogeneity of variances (Levene-test). In the case of histopathological data, the Kruskal–Wallis analysis and Chi-Square test were used for the evaluation of the mean score and ratio of affected animals, respectively. Significance was determined at $P \leq 0.05$. Statistical analysis was performed with SPSS 20.0 for Windows software package.

2.3. Effect of dietary supplementation of milk thistle seed oil and a symbiotic feed additive on some performance, carcass, digestive physiology and antioxidant parameters

2.3.1. Experimental animals and dietary treatments

The experiment was conducted at the experimental farm of the Institute of Physiology and Nutrition of the Hungarian University of Agriculture and Life Sciences (Georgikon Campus, Keszthely). The experiment complied with the Government Decree 40/2013. (II. 14.) on animal experiments and approved by the Animal Welfare Committee of MATE Georgikon Campus (MÁB-2/2017). A total of 240, day-old Cherry Valley SM3 hybrids were purchased from the hatchery of Hungerit Co.

(6600 Szentes, Attila út 3.). Animals were housed in deep litter floor pens and randomly divided into four treatment groups with six pens per treatment (n = 60/treatment, n = 10/pen). The animals did not receive any pharmacological treatments or vaccines during the experiment. Chopped wheat straw was used as bedding material. The useful floor area of the pens was 1.75 m^2/pen, so the stocking density corresponded to 5.71 animals/m^2. The temperature, humidity and ventilation parameters of the room were controlled according to the needs of the hybrid using an automatic, computer-controlled system during the entire duration of the experiment. In order to ensure an optimal temperature for the young ducks, an infrared lamp was used in each pen. On placement day, the average temperature under the infrared lamps was 32-33°C, while in the room it was 30°C, which was gradually reduced according to age requirements. From the 22nd day until the end of the experiment, a constant temperature of 18°C was set measured at the level of the animals. Drinking water was provided by bell drinkers and diets were available in hand-filled feeders. Water and feed were available to the ducks *ad libitum*. The experimental diets of the four treatments were fed in three phases (starter: day 0-9, grower: day 10-16, finisher: day 17-43) during the 43-day-long fattening experiment.

The experimental dietary treatments were as follows: 2% sunflower oil supplementation without symbiotic (A), 2% milk thistle seed oil supplementation without symbiotic (B), 2% sunflower oil supplementation with 1 g/kg symbiotic (C), 2 % milk thistle seed oil supplement + 1 g/kg symbiotic (D). Commercially available Floriol refined sunflower cooking oil and cold-pressed milk thistle seed oil from Natúr Press Team Ltd. (2948 Kisigmánd Újpuszta 0195/1 Hrsz) were used for diet preparation. The commercially available symbiotic called Poultry Star® (Biomin GmBH, Austria) contained prebiotic fructooligosaccharides and bacterial strains (*Bifidobacterium, Lactobacillus, Enterococcus*) isolated from the intestinal contents of SPF chickens. The calculated nutrient content of experimental diets met the requirements of the breed. During the five days before the sampling, the experimental animals

consumed diets containing 0.5% TiO_2 indicator. In order to add 0.5% of the indicator, the corn concentration of the final feed was reduced by 0.5%. The silymarin content of milk thistle seed oil was 164 μg/ml on average in two samples, measured by Bálint Analitikai Ltd. (Fehérvári út 144, 1116 Budapest).

2.3.2. Measurement of production parameters

The individual body weight of ducks was measured on 0, 9, 16 and 43 days of the experiment, and the body weight gain for the periods between the measurement days and the entire experiment was calculated from the obtained results. Feed intake per pen and per treatment group was measured for the starter, grower and finisher phases. Based on the body weight gain and feed intake data, the feed conversion ratio was calculated for the dietary phases and for the whole experiment.

2.3.3. Sampling

On day 43, 2 ducks from 5 pens per treatment, i.e., 10 ducks per treatment, and a total of 40 ducks were randomly selected for sample collection. After weighing the body weights, the animals were stunned with carbon dioxide gas and slaughtered gently. After opening the body cavity, the breast fillet, thighs and liver were removed and weighed. The liver samples collected from the right lobe were immediately placed in liquid nitrogen and stored at -80°C until the antioxidant parameter analysis. Samples of the intestinal content were collected individually from the jejunum, ileum and caecum. The samples from the jejunum were collected from the proximal part of the jejunum, which is approximately 50% of the total length of the jejunum. Ileal samples were collected from the intestinal section between the caecum and Meckel's diverticulum. The samples of the intestinal contents were stored at -20°C until the analyses.

2.3.4. Analytical methods

2.3.4.1. Analyses of liver samples

The antioxidant parameters of the liver samples were measured in the laboratory of the MATE ÉTI Department of Food Safety. The reduced glutathione (GSH) concentration was measured from the 10,000 g supernatant fraction of a 1:9 homogenate prepared from liver tissue (Sedlak and Lindsay, 1968). In order to remove the protein sulfhydryl groups, the reaction was carried out after protein precipitation with a 10% (w/v) TCA solution. The reagent forming a colored complex with non-protein sulfhydryl groups was 5,5'-dithiobis-(2-nitrobenzoic acid). It shows an absorption maximum at 412 nm after adjustment to pH 8.0-8.2 with Tris buffer (pH: 8.9) and can be measured spectrophotometrically. The quantitative determination was based on the value of the molar extinction coefficient of GSH determined in the given system (E1cm 1% = 13100).

The activity of the glutathione peroxidase (GSHPx) enzyme was measured in the 10,000 g supernatant fraction of a 1:9 homogenized liver tissue (Matkovics et al., 1988). The principle of the method is that GSH dimerizes into glutathione disulfide (GSSG) induced by an oxidative enzymatic reaction in the presence of oxygen free radicals. The measurement was performed in the presence of cumene hydroperoxide (CHPO) and GSH co-substrates, where the appropriate pH-value was adjusted with Tris buffer (pH 7.6). The incubation time was 10 minutes at room temperature and the reaction was stopped with a 10% (w/v) TCA solution. The sulfhydryl reagent 5,5'-dithiobis-(2-nitrobenzoic acid) was used to measure the oxidation of glutathione, which forms a yellow complex with the remaining reactive SH groups and can be measured spectrophotometrically at 412 nm after adjusting the pH to 8.0-8.2 with Tris buffer.

The concentration of thiobarbituric acid-reactive malondialdehyde (MDA) was measured in the native homogenate of liver tissue (Placer et al., 1966; Matkovics et

al., 1988). The principle of the measurement is that malondialdehyde forms a yellowish-red complex with 2-thiobarbituric acid at 100°C in 20 minutes in an acidic medium and can be measured spectrophotometrically with an absorption maximum at 535 nm wavelength. The protein concentration of the liver tissue homogenates was measured with the Folin-phenol reagent based on the color reaction of the 10,000 g supernatant fraction, where bovine serum albumin served as a standard (Lowry et al., 1951).

2.3.4.2. Analyses of intestinal content and feed samples

The activity of some pancreatic enzymes, such as α-amylase, lipase and trypsin, was determined from the samples of jejunal content using a spectrophotometric method. The α-amylase activity was determined by the method of Dahlqvist (1962), while the lipase activity was determined using the method developed by Schön et al. (1961). Trypsin activity was measured using the Boehringer test, based on the method of Kakade et al. (1969).

The crude fat (MSZ EN ISO 6492), crude fiber (MSZ EN ISO 6865: 2001), total phosphorus (MSZ EN ISO 6491: 2001) and calcium (MSZ EN ISO 6869: 2001) contents of the experimental diets were also measured. The content of dry matter (MSZ EN ISO 6496:2001), crude protein (MSZ EN ISO 5983-2:2009), and some amino acids (lysine, methionine, threonine, arginine, histidine , phenylalanine, proline, glycine, valine, leucine, isoleucine, cysteine, aspartic acid, serine, glutamic acid, alanine, tyrosine) and the concentration of TiO_2 were determined in ileal chymus and feed samples. The concentration of amino acids was measured with an automatic amino acid analyzer (Ingos Amino Acid Analyzer AAA 400; Ingos, Czech Republic). Before the determination, the samples were oxidized in formic acid to prevent the loss of methionine and cystine content. After that, acidic hydrolysis was performed with the samples for 24 hours, at a temperature of 110 °C, with a 6M HCl solution. The TiO_2 concentration was determined using a Libra S12 UV-VIS spectrophotometer, measured at a wavelength of 410 nm using the method of Short et al.

(1996). The apparent ileal digestibility of amino acids was calculated based on the following equation. Amino acid digestibility (%) = {(IAAb – IAAt) / IAAb}x100; where IAAb: indicator/amino acid ratio in intestinal contents; IAAt: indicator/amino acid ratio in feed.

The short-chain fatty acid (SCFA) content was determined from the mixed contents of the right and left intestinal sections of the cecum using the gas chromatography method (TRACE 2000, Thermo Scientific, Waltham, MA, USA). The frozen samples were thawed and mixed them thoroughly. After that, 250 µl of intestinal content was mixed with 600 µl of 1.11 M hydrochloric acid. The gas chromatograph was equipped with a 30 m (0.25 mm inner diameter) fused silica gel column (Nukol column, Supelco Inc., Bellefonte, PA, USA). A flame ionization detector (FID) was used with a split (1:50), where the injection volume was 1 µL at 220 °C and the detection was done at 250 °C. The carrier gas was helium at a pressure of 83 kPa. For calibration, mixtures of standard SCFAs (1, 4, 8, and 20 mM) were used containing acetate, propionate, n-butyrate, and n-valerate as external standards.

To determine the fatty acid composition of diets and oils, total fat content was extracted and lipid extracts were converted to fatty acid methyl esters with BF3-methanol (AOAC, 1990). Fatty acid methyl esters were separated and analyzed by gas chromatography using an Omegavax 320 capillary column (30 m length x 0.32 mm ID, 0.25 µm film) in a TRACE 2000 gas chromatograph (TRACE 2000, Thermo Scientific, Waltham, MA, USA). The temperature of the furnace and flame ionization detector was set to 200 and 260 °C, respectively. Helium was used as carrier gas (25 cm/s, set at 200 °C) and the split ratio was 100:1. The identification of individual fatty acids was performed by comparison with a known standard mixture of fatty acid methyl esters (PUFA-2, Supelco catalog number: 4-7015-U; Supelco, Bellefonte, PA, USA).

2.3.5. Statistical methods

The data were evaluated using two-way analysis of variance and Tukey's post hoc test after examining the normal distribution of the data (Kolmogorov-Smirnov test) and homogeneity of variances (Levene test). Significance was defined as $P \leq 0.05$. The statistical analysis was performed with the SPSS 20.0 for Windows software package.

3. Results and discussion

During my *in vitro* experiment, when applying both concentrations of milk thistle seed cake extract and milk thistle oil (0.5 and 1.5 g/100 ml medium), the logCFU values of total and faecal *coliforms* and *Enterococcus* bacteria were not significantly different compared to the control treatment (P>0.05). On the other hand, the results tended to show a small antimicrobial effect of the treatments on the examined facultative pathogenic bacterial groups. At the same time, when examining *Lactobacillus* strains, the dose of 1.5 g/100 ml of milk thistle seed cake extract and both concentrations of milk thistle seed oil resulted in significantly higher logCFU values of *Lactobacillus* strains compared to the control group (P <0.05). The inhibition rates calculated on the basis of relative logCFU values compared to the control group (100%) are shown in Table 1 for each treatment.

Table 1. The effect of experimental treatments on the inhibition rate of certain bacterial groups (%).

Treatments	Conc. in the media	Coliform (total)	Faecal coliform	Enterococcus	Lactobacillus
Control	0g/100 ml	0	0	0	0
Milk thistle seed extract	0.5 g/100 ml	2.5	3.1	1.7	0.4
	1.5 g/100 ml	11.5	11.4	8.0	-20.4
Milk thistle seed oil	0.5 g/100 ml	4.1	3.7	2.2	-17.1
	1.5 g/100 ml	6.9	9.2	8.3	-16.7

Based on the results of my experiment, it is likely that *Lactobacillus* species, which are present in larger numbers due to the active ingredients of milk thistle, can exert an inhibitory effect on facultative pathogens through competition and production of lactic acid, which lowers the pH of the intestinal contents.

During my second experiment, the body weight of the ducks in the group consuming diets with milk thistle seed oil was significantly lower on day 42 compared to the control animals (P<0.05; Table 2). The relative liver weight of the ducks was not significantly affected by milk thistle treatments (Table 2). On day 28, the relative

weight of the spleen was significantly lower in the milk thistle seed cake group compared to the control group, while the relative weight of the bursa of Fabricius was also significantly lower in this group compared to the group receiving milk thistle seed oil supplementation ($P<0.05$). By the end of the experiment, the significant differences observed in the case of the spleen and the bursa of Fabricius were not proved, the relative weight of the organs in the milk thistle treatment groups did not differ compared to the control group.

Table 2. The effect of the experimental treatments on the body weight (g) of the ducks and the relative weight of the liver (%)

	Treatments	Day 14		Day 28		Day 42	
		Mean	SEM	Mean	SEM	Mean	SEM
Body weight (g)	A	313.60	15.75	1160.40	66.21	2393.00 [a]	80.23
	B	307.60	23.18	1184.80	38.40	2102.75 [b]	42.53
	C	268.40	23.99	1090.40	40.53	2262.00 [ab]	52.54
	D	355.20	32.61	1216.00	29.83	2152.50 [ab]	52.42
	Significance	*NS*		*NS*		*P = 0.044*	
Relative weight of the liver (%)	A	4.55	0.42	2.73	0.20	2.72	0.06
	B	4.15	0.07	2.79	0.15	2.34	0.09
	C	4.55	0.32	2.54	0.11	2.54	0.09
	D	4.12	0.22	2.74	0.11	2.67	0.16
	Significance	*NS*		*NS*		*NS*	

A: control, B: 0.1% milk thistle oil, C: 0.5% milk thistle seed cake, D: 0.5% milk thistle seed; NS: non-significant ($P > 0.05$). [ab] Means in the same column with different superscripts are significantly different ($P < 0.05$).

Of the measured clinical chemical parameters, only the serum creatinine concentration was significantly affected by the experimental treatments, and no verifiable differences were observed for the other parameters (AST, ALT, glucose, cholesterol, triglycerides, uric acid). Table 3 shows the effect of the experimental treatments on the histopathological results of the liver, spleen and bursa of Fabricius. The milk thistle treatments used during my experiment effectively reduced the incidence rate and severity of the observed histopathological changes.

Table 3. The effect of experimental treatments on the histopathological parameters of the liver, spleen, and bursa of Fabricius

	Treatments	Day 14		Day 28		Day 42	
		Mean score	Ratio (%)	Mean score	Ratio (%)	Mean score	Ratio (%)
Liver							
vacuolar cell degeneration	A	2.6 [a]	100.0	1.2	40.0	2.4 [a]	100.0 [a]
	B	1.0 [b]	60.0	0.4	40.0	0.6 [b]	50.0 [b]
	C	2.0 [ab]	100.0	1.0	80.0	1.4 [ab]	62.5 [a]
	D	1.0 [b]	60.0	0.6	40.0	1.6 [ab]	100.0 [a]
	Significance	*P=0.026*	NS	NS	NS	*P=0.001*	*P=0.001*
solitary cell death	A	0.0	0.0	1.0 [a]	100.0 [a]	0.1	12.5
	B	0.0	0.0	0.0 [b]	0.0 [b]	0.0	0.0
	C	0.0	0.0	0.4 [ab]	40.0 [b]	0.0	0.0
	D	0.0	0.0	0.0 [b]	0.0 [b]	0.0	0.0
	Significance	*NS*	*NS*	*P=0.032*	*P=0.030*	*NS*	*NS*
cell death of mononuclear phagocyte svstem	A	0.0	0.0	0.4	40.0	0.1	12.5
	B	0.0	0.0	0.0	0.0	0.0	0.0
	C	0.0	0.0	0.2	20.0	0.0	0.0
	D	0.0	0.0	0.0	0.0	0.0	0.0
	Significance	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*
infiltration of lympho- and histiocytes	A	0.0	0.0	1.6 [a]	80.0 [a]	1.1	87.5
	B	0.2	20.0	0.4 [ab]	40.0 [b]	1.1	87.5
	C	0.0	0.0	0.2 [b]	20.0 [b]	0.9	62.5
	D	0.2	20.0	0.0 [b]	0.0 [b]	0.9	75.0
	Significance	*NS*	*NS*	*P=0.001*	*P=0.001*	*NS*	*NS*
interstitial fi-brosis	A	0.0	0.0	1.6 [a]	100.0 [a]	1.1	87.5 [a]
	B	0.0	0.0	0.0 [b]	0.0 [b]	0.3	12.5 [b]
	C	0.0	0.0	0.0 [b]	0.0 [b]	0.9	62.5 [a]
	D	0.0	0.0	0.0 [b]	0.0 [b]	0.6	50.0 [a]
	Significance	NS	NS	*P=0.026*	*P=0.026*	NS	*P=0.033*
Spleen							
decreased lymphocyte count	A	1.0 [a]	100.0 [a]	0.0	0.0	0.0	0,0
	B	0.0 [b]	0.0 [b]	0.0	0.0	0.0	0,0
	C	0.0 [b]	0.0 [b]	0.0	0.0	0.0	0,0
	D	0.0 [b]	0.0 [b]	0.0	0.0	0.0	0,0
	Significance	*P=0.001*	*P=0.001*	*NS*	*NS*	*NS*	*NS*
Bursa of Fabricius							
decreased lymphocyte count	A	1.0 [a]	100.0 [a]	0.0	0.0	0.0	0,0
	B	0.0 [b]	0.0 [b]	0.0	0.0	0.0	0,0
	C	0.0 [b]	0.0 [b]	0.0	0.0	0.0	0,0
	D	0.0 [b]	0.0 [b]	0.0	0.0	0.0	0,0
	Significance	*P=0.001*	*P=0.001*	*NS*	*NS*	*NS*	*NS*

A: control, B: 0.1% milk thistle oil, C: 0.5% milk thistle seed cake, D: 0.5% milk thistle seed; NS: non-significant ($P > 0.05$). [ab] Means in the same column with different superscripts are significantly different ($P < 0.05$).

The most pronounced histopathological change in the liver caused by the mycotoxins was vacuolar degeneration of hepatocytes, which was significantly lower in the groups receiving milk thistle oil and milk thistle seed supplementation on day 14 and on day 42 compared to the control group ($P<0.05$). Based on my results, all three milk thistle treatments contributed almost equally effectively to reducing the proportion of animals affected by solitary liver cell death and lymphocytic infiltration on day 28 of the experiment. All milk thistle treatments successfully prevented the decrease of lymphocyte count in the bursa of Fabricius and the spleen, where the lesion was mildly present in all animals of the control group on day14.

During my third experiment, none of the dietary treatments consisting of milk thistle seed and sunflower oil, or their addition with symbiotic, significantly affected the body weight (Table 4) and other performance characteristics of the ducks ($P>0.05$).

Table 4. The effect of feed treatments on the body weight of experimental animals (mean ± SEM)

Dietary treatments		Body weight (g)		
Oil supplement	Symbiotic	Starter phase (day 9)	Grower phase (day 16)	Finisher phase (day 43)
Sunflower oil	S-	238.2 ± 4.9	716.7 ± 12.6	3426.0 ± 34.6
	S+	229.2 ± 6.3	687.4 ± 16.3	3404.2 ± 45.3
Milk thistle seed oil	S-	233.9 ± 5.5	695.8 ± 13.6	3402.4 ± 40.1
	S+	239.5 ± 5.1	669.7 ± 12.1	3408.5 ± 32.6

S -/+ = without symbiotic/with symbiotic

At the end of the experiment, no significant differences were observed between the control and treated groups in the absolute or relative weight of the breast fillet, thighs and liver, similar to the performance parameters. None of the experimental treatments affected significantly the activity of three pancreatic enzymes in the jejunal intestinal content, α-amylase, lipase and trypsin, or the ileal apparent digestibility of amino acids.

In the case of the short-chain fatty acid concentration of the caecal contents, the oils used did not exert a significant influence on any of the fatty acids examined. Among the examined fatty acids, a significant symbiotic effect was demonstrated only in the case of propionic acid, where the use of symbiotic significantly reduced the concentration of propionic acid in the caecal content ($P<0.05$).

The experimental treatments did not result in significant differences in the averages of the most important parameters characterizing the antioxidant system (GSH, GSHPx, MDA) of the liver tissue samples based on the effect of the oil type or the symbiotic supplement. The measured antioxidant parameters are listed in Table 5.

Table 5. The effect of dietary treatments on the antioxidant parameters of liver (mean ± SEM)

Dietary treatments		Parameters		
Oil supplement	Symbiotic	GSH (μmol/g prot.)	GSHPx (E/g prot.)	MDA (nmol/g)
Sunflower oil	S-	9.14 ± 0.11	8.00 ± 0.58	32.18 ± 1.07
	S+	10.94 ± 0.97	10.07 ± 0.97	27.51 ± 4.31
Milk thistle seed oil	S-	10.07 ± 0.56	9.33 ± 0.81	24.75 ± 2.90
	S+	8.35 ± 0.13	6.51 ± 0.27	26.89 ± 7.82
Effect of oil supplementation				
Sunflower oil		9.65 ± 0.33	8.46 ± 0.49	31.28 ± 1.92
Milk thistle seed oil		9.26 ± 0.33	7.65 ± 0.51	27.03 ± 1.87
Effect of symbiotic supplementation				
S-		9.44 ± 0.33	8.21 ± 0.49	28.16 ± 1.92
S+		9.47 ± 0.33	7.90 ± 0.51	30.15 ± 1.87
Level of significance				
Oil		NS	NS	NS
Symbiotic		NS	NS	NS
Oil + Symbiotic		NS	NS	NS

S -/+ = without symbiotic/with symbiotic; NS = non-significant (P>0.05)

4. Conclusions and recommendations

Based on the results of my *in vitro* experiment, it can be concluded that milk thistle seed cake extract and milk thistle seed oil are able to slightly inhibit the growth of Gram-negative *coliform* and Gram-positive *Enterococcus* facultative pathogenic strains belonging to the *Enterobacteriaceae* family. However, this effect is dose-dependent, and the higher concentration (1.5 g/100 ml medium) resulted in more favourable inhibition percentages. From the point of view of *Lactobacillus* species, active substances of milk thistle resulted in a negative inhibition (-16.7-20.6%), which means an effect supporting reproduction. The demonstrated effect of the flavonolignan complex found in milk thistle can also be beneficial *in vivo* in terms of the composition of the microbiota. If it can support the activity of lactic acid-producing bacteria, they can contribute to inhibiting the proliferation of facultative pathogenic species. Although the colony-forming unit of *Bifidobacteria* was not determined during my experiment, based on the mechanism of action of the polyphenol-like flavonolignans, it can be assumed that we can have a favourable effect on the reproduction of these microbes by using milk thistle-based feed supplements in the appropriate concentration. Based on my results, milk thistle supplements can contribute to preventing the development of dysbiosis, and thereby reducing the use of antibiotics. Based on the *in vitro* experiment, I would also recommend conducting an *in vivo* experiment, in which the effect of the active ingredients of milk thistle on facultative pathogenic bacteria could be observed by examining the composition of the intestinal microbiota. In this experiment, an important research goal could also be to investigate the possible stimulatory effect of milk thistle on the activity of *Lactobacillus* and *Bifidobacterium* species - observed during the in vitro test.

In my second, *in vivo* experiment, the contamination of the feed with DON and ZEN mycotoxins did not cause clinical symptoms, did not affect the development of some important organs and the body weight of the experimental animals. Based on the

clinical-chemical parameters of the examined blood serum, the mycotoxin contamination of the diets did not result in serious liver and kidney damage. The change in the activity of the ALT and AST enzymes shows a significant increase in cases of acute damage (severe toxicosis, inflammation), while during chronic processes - such as in our experiment, - the continuous intake of mycotoxins in tolerated amounts leads to a slight increase or no significant change of the enzyme activity. The active ingredients of milk thistle are proven to have liver and kidney protective effects, but in our experiment, due to the lack of expressed negative effects of the tested mycotoxins, we could not observe these effects in the clinical chemical parameters of the blood serum. In my study, the hepatoprotective effect of milk thistle seed, milk thistle cake and milk thistle oil supplemented to diets fed from the beginning of the rearing period was proved with histopathological examinations. The changes observed during my examination were of medium and mild degree, and were present in a higher proportion and average score in the animals of the control group. There were no adverse changes in the lymphoid organs in the milk thistle treatment groups in contrast to the control group, where lymphocyte depletion was mild. The positive effects of milk thistle are also important and useful in cases that do not manifest in clinical symptoms which often occur in large-scale practical conditions. In these cases, the histological and functional integrity of the examined organs have high importance from the point of view of the metabolism, product quantity and quality, and the functioning of the immune system. Milk thistle supplements are supposed to protect hepatocytes and cells of lymphoid organs even in case of more severe mycotoxin contamination. Based on the results of the experiment, I would recommend further investigation of diets contaminated with DON and ZEN in ducks in such a way that the degree of contamination exceeds the doses tolerated by the ducks. In addition to determining the histopathological and clinical chemical parameters of the blood serum, it would also be worthwhile to examine indicators of the body's antioxidant system.

During the third experiment, the effect of diets supplemented with either 2% milk thistle seed oil or 2% sunflower oil, alone or with a symbiotic supplement containing fructooligosaccharides and three types of bacteria were examined. Milk thistle seed oil did not affect the performance and carcass parameters of ducks, the activity of pancreatic enzymes, the concentration of short-chain fatty acids in the caecal contents (except for propionic acid), the apparent ileal digestibility of amino acids, the indicators of the antioxidant system of liver (GSH, GSHPx, MDA), even without and with the symbiotic. In my opinion, harmful technological and health factors were not present in the experiment, which would probably have shown the protective nature of the milk thistle. The results of the experiment show that milk thistle seed oil can be used with the same efficiency as sunflower oil in diets of duck fattening. Based on all of this, I would like to make a proposal for further studies in order to gain a more thorough understanding of the effects of milk thistle seed oil, in which different, stressful so-called "challenge" effects could be investigated. Diets contaminated with mycotoxins, heat stress, and some infectious diseases (e.g. hepatitis virus infection) can be challenging factors, whose harmful effects could be reduced by milk thistle seed oil. These experiments can be combined with vaccination, when the effectiveness of the immunization can be tested in addition to a possible challenging effect (e.g. mycotoxin contamination). Also, I would recommend carrying out histopathological examinations in all cases to see the possible beneficial effects of the active ingredients of milk thistle in the long term. As a further research opportunity, I would recommend investigating the liver protective effects of different milk thistle products in aged egg-producing poultry flocks and with ducks for liver production, because in their case the metabolism of the liver is extremely stressed, and fatty liver can be considered as a physiological phenomenon.

5. New scientific results

1. Milk thistle seed cake extract (1.5g/100 ml medium) and milk thistle seed oil (0.5 and 1.5g/100 ml medium) can significantly promote the growth of *Lactobacillus* bacteria in the case of samples from the ileal contents of ducks using the *in vitro* agar culture method ($P<0.05$).

2. Supplementing the diets with milk thistle seed cake (0.5%) and milk thistle seed oil (0.1%) has a beneficial effect on some histopathological parameters of the liver of ducks (vacuolar degeneration, solitary liver cell death, interstitial lympho- and histiocyte infiltration of connective tissue, interstitial fibrosis) fed diets contaminated with deoxynivalenol and zearalenone mycotoxins.

3. The addition of milk thistle seed (0.5%), milk thistle seed cake (0.5%) and milk thistle seed oil (0.1%) to the diets can reduce the levels of lymphocyte depletion in the spleen and bursa of Fabricius of ducks consuming diets contaminated with the mycotoxins deoxynivalenol and zearalenone.

4. Supplementing the diets with 2% milk thistle seed oil without or with the use of a symbiotic (fructooligosaccharides and strains of *Bifidobacterium*, *Lactobacillus*, *Enterococcus* bacteria) does not affect the performance (feed intake, body weight gain, feed conversion ratio) and carcass (breast fillet, the relative weight of thighs and liver) characteristics of ducks; the activity of pancreatic (α-amylase, lipase, trypsin) enzymes, the apparent ileal digestibility of essential (except tryptophan) and some non-essential amino acids, as well as the concentrations of short-chain fatty acids in the cecal contents, and also the most important antioxidant parameters of the liver (reduced glutathione level, activity of glutathione-peroxidase enzymes, malondialdehyde concentration) in comparison with a diet supplemented with 2% sunflower oil.

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7. Publications related to the topic of the dissertation

I. Publications in peer-reviewed journals in foreign language:

Bencze-Nagy J., Strifler P., Horváth B., Such N., Farkas V., Dublecz K., Pál L. (2023): Effects of dietary milk thistle (*Silybum marianum*) supplementation in ducks fed mycotoxin-contaminated diets. Veterinary Sciences, 10 (2) 100. (Q1, IF: 2,51)

II. Publications in peer-reviewed journals in Hungarian:

Bencze-Nagy J., Such N., Koltay I. A., Molnár A., Farkas V., Dublecz K., Rózsa L., Pál L. (2020): A máriatövis (*Silybum marianum*) egészségvédő hatásai. Magyar Állatorvosok Lapja, 142 (4) 229-240. (Q4, IF: 0,08)

Nagy J., Pál L., Rózsa L. (2018): Máriatövis felhasználási lehetőségei a gazdasági állatok takarmányozásában. Animal Welfare, Etológia és Tartástechnológia, 14 (2) 78-91.

III. Publications in full length in a peer-reviewed conference proceedings:

Nagy J., Sipiczki B., Fébel H., Rózsa L., Molnár A., Pál L. (2017): Máriatövis antimikrobiális hatásának *in vitro* vizsgálata. LIX. Georgikon Napok, szeptember 28-29. Keszthely. (teljes anyag: http://napok.georgikon.hu) ISBN 978-963-9639-88-1.

IV. Publications in abstract form in a peer-reviewed conference proceedings:

Molnár A., Pál L., Farkas V., Menyhárt L., Bató E., Bihari Z., **Nagy J**., Husvéth F., Dublecz K. (2018): A symbiotic supplement results in a propionic acid decline and limited microbiota shift in duck cecal content. The XVth European Poultry Conference, 17-21st September, Dubrovnik, Croatia. p. 322.

Such N., Koltay I. A., **Nagy J**., Szűcs K., Molnár A., Pál L., Wágner L., Husvéth F., Dublecz K. (2018): A mezőgazdasági területeke antibiotikum szennyezésének kockázata a szerves trágyán keresztül, hatásaik a növényekre, kiváltási lehetőségeik az állati termelésben. Tavaszi Szél Konferencia 2018, Nemzetközi Multidiszciplináris Konferencia, Széchenyi István Egyetem, május 3-6., Győr. Absztraktkötet, 66. oldal.