agriculture

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Influence of Effective Microorganisms and Clinoptilolite on Gut Barrier Function, Intestinal Health and Performance of Broiler Chickens during Induced *Eimeria tenella* Infection

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Abstract: The prohibition of certain coccidiostats in poultry has created a need to seek an alternative to control *Eimeria* infection. The aim of this study was to evaluate the effects of effective microorganisms (EM) in a multi-strain probiotic (Bokashi®), with clinoptilolite as a feed supplement on the mRNA expression of tight junction proteins and redox enzymes in the caecal tissue of chickens infected with *E. tenella*. The integrity of the intestinal barrier was tested by determining the concentration of fluorescein isothiocyanate dextran (FITC-d) in the chicken's serum. A total of 600 1-day-old Ross 308 male chickens received diets with a 0.5% or 0.8% concentration of the probiotic together with clinoptilolite. The experiment used 5 treatment groups, and a control group, each with 5 replicates with 20 birds. The results indicate that the use of the 8 kg/t of feed multi-strain probiotic together with clinoptilolite in the diet of poultry caused a significant reduction in the number of *E. tenella* oocysts in the faeces and caecum and significantly improved the growth rate of chicken broilers infected with *E. tenella*. In addition, the probiotic and clinoptilolite enhanced antioxidant processes in the caecal mucosa and reduced oxidative stress induced by *E. tenella* infection.

Keywords: chicken; multi-strain probiotic; clinoptilolite; *Eimeria tenella*; tight junction proteins

Citation: Ciszewski, A.; Jarosz, Ł.S.; Kalinowski, M.; Marek, A.; Grądzki, Z.; Grabowski, S.; Hejdysz, M.; Nowaczewski, S.; Rysiak, A. Influence of Effective Microorganisms and Clinoptilolite on Gut Barrier Function, Intestinal Health and Performance of Broiler Chickens during Induced *Eimeria tenella* Infection. *Agriculture* **2022**, *12*, 2176. https://doi.org/10.3390/agriculture12122176

Academic Editor: Zhigang Song

Received: 3 November 2022
Accepted: 15 December 2022
Published: 19 December 2022

1. Introduction

One of the most important health and economic problems in intensive poultry production is coccidiosis, caused by infection with *Eimeria tenella* [1,2]. This protozoon is found in the caecal mucosa, where it damages epithelial cells and leads to the development of bloody diarrhoea and malabsorption, resulting in a slower growth rate, poorer feed conversion, and ultimately increased mortality [3,4]. At the subcellular level, *E. tenella* damages tight junctions (TJ) in the intestinal barrier, which are an important element regulating mucosal permeability and the integrity of the intestinal epithelium [5–8]. Impairment or loss of intercellular junctions caused by this protozoon, as a consequence of changes in TJ structure, adversely affects the selective permeability of the intestines, responsible for passive transport of small water-soluble molecules, and leads to reduced nutrient absorption and utilisation [8,9]. Disturbances of the integrity of the epithelium of the intestinal barrier during *E. tenella* infection also lead to the impairment of GALT (gut-associated lymphoid tissue) activity and stimulate the release of pro-inflammatory cytokines and the expression of numerous proteins taking part in the immune response to infection, which is conducive to the development of intestinal inflammation [10,11]. Moreover, *E. tenella* affects

the composition of the caecal microbiome of birds, decreasing the number of saprophytic bacteria and increasing the number of conditionally pathogenic bacteria, which leads to dysbacteriosis and disturbances of the fermentation of the digesta in the caecum, creating conditions favourable to the development of inflammation [4,12].

E. tenella infection and the spread of coccidiosis in poultry flocks are currently prevented by conventional methods, mainly chemoprophylaxis and immunoprophylaxis [13]. The emergence of drug-resistant *E. tenella* strains in the breeding environment and the ban on the use of certain coccidiostats, as well as the occurrence of subclinical post-vaccination *Eimeria* infections, have created the need for new, alternative strategies to control these infections in poultry [14]. Modern feeding strategies based on the use of essential oils, pro-, pre- and synbiotics, or various plant extracts make it possible to prevent or mitigate the negative effects of *E. tenella* infection in broilers by stimulating intestinal epithelial cells, modulating the intestinal microbiome, and stimulating GALT mechanisms [15–17].

Effective microorganisms (EM) and clinoptilolite (zeolite) are increasingly used in poultry production to stabilise the intestinal microbiome, improve digestion and nutrient absorption, and increase the immunity of birds. Effective microorganisms (EM) are widely used in livestock farming, e.g., as feed additives that regulate intestinal function by stabilising and maintaining the microbial balance between pathogenic and saprophytic microbes [18]. EM also takes part in the intestinal digestion of proteins, carbohydrates, and fats, stimulates the synthesis of biologically active compounds, including enzymes and vitamins, and is involved in detoxification processes [19,20]. The beneficial effects of EM in poultry production are manifested as increased daily weight gains, improved feed absorption, increased production performance in birds, and decreased mortality rates [21,22]. Aluminosilicates, which include clinoptilolite, are hydrated volcanic rock soils used to ensure good sanitary conditions and as feed additives to improve growth performance and meat quality [23]. Natural clinoptilolite is a hydrated aluminosilicate. Hydrated aluminosilicates have ion-exchange and adsorption properties, and when added to litter and feed, they adsorb ammonia and mycotoxins, thereby improving the quality and biosecurity of poultry production [24,25]. Numerous studies have shown that clinoptilolite used as a dietary supplement for cattle, pigs, and poultry increases the digestibility of feed nutrients, which improves productivity and reduces susceptibility to disease. Clinoptilolite has also been shown to influence the morphology of intestinal cells in broiler chickens [26] and to modify the composition of the gastrointestinal microbiome [27–29]. Moreover, clinoptilolite improves digestion and absorption in rats, lambs, pigs, and laying hens [28,30–32] and increases weight gains and feed conversion [31], thereby reducing production costs. The mechanisms of action of probiotics containing effective microorganisms and clinoptilolite on birds are complex and not yet fully understood, especially with regard to infection with *Eimeria* spp. Previously published research indicates that probiotic bacteria favourably influence the functions of the intestinal barrier by maintaining paracellular permeability, increasing the production of mucus coating the enterocytes, stimulating the immune system, and modulating the composition of the intestinal microbiome [33]. In vitro studies have shown that *Enterococcus faecium* decreases the permeability of the intestinal epithelium by increasing the expression of transmembrane proteins that form tight junctions (TJ) [34]. Similar observations have been made by Chang et al. [35], who demonstrated that a multi-strain probiotic including *Lactobacillus acidophilus* LAP5, *Lactobacillus fermentum* P2, *Pediococcus acidilactici* LS, and *Lactobacillus casei* L21 used as a feed additive in poultry diets increased the mRNA expression of OCLDN (occludin), ZO1 (zonulin), and MUC (mucin). The increased concentrations of these proteins inhibited the multiplication and harmful effects of pathogenic *Salmonella* bacteria by increasing mucus production, which prevents bacteria from adhering to enterocytes and ensures the integrity of epithelial cells and tight junctions (TJ). A similar effect is achieved in poultry through dietary supplementation with clinoptilolite, which has a strong capacity to adsorb intestinal bacteria, toxic substances, and other harmful compounds [28,29]. In addition, clinoptilolite causes morphological abnormalities in sporulated oocysts, which collapse and disintegrate. These processes

reduce the secretion of sporulated oocysts to the environment and thus their infectious potential [36].

Little is known of the effect of formulations combining effective microorganisms (EM) and clinoptilolite on the intestinal barrier of birds in the case of simultaneous infection with *E. tenella*. So, we made the hypothesis that a high-quality feed supplement based on a mixture of effective microorganisms (EM) and clinoptilolite could be used to combat *E. tenella* infection. The aim of this study was to evaluate the mRNA expression of OCLDN (occludin), CLDN1 (claudin 1), CLDN2 (claudin 2), ZO1 (zonula occludens 1), ZO2 (zonula occludens 2), JAM2 (junctional adhesion molecule 2), and MUC2 (intestinal mucin 2) in the caecal tissue of chickens infected with *E. tenella* and supplemented with feed additives containing effective microorganisms (EM) and clinoptilolite. In addition, the mRNA expression of redox enzymes SOD (superoxide dismutase 1), CAT (catalase), and HMOX1 (haem oxygenase 1) was assessed, as well as growth performance and health parameters as indicators of the profitability of production.

2. Materials and Methods

2.1. Experimental Animals

The experiment was conducted at the Experimental Station of the Poznan University of Life Sciences, Gorzyń 4, Międzychód commune. Consent for all research procedures was obtained from the Local Ethics Committee for Animal Testing at the University of Life Sciences in Lublin, Poland (approval no. 11/2021 of 1 March 2021).

A total of 600 1-day-old Ross 308 male chickens were used in the experiment. There were 6 treatments, each of which had 5 replicates with 20 birds per replicate pen. The treatments were as follows: a basal diet (control group—group I); basal diet + 0.5% multi-strain probiotic formulation EM Bokashi® per tonne of feed + 3% clinoptilolite (group II); basal diet + 0.8% multi-strain probiotic formulation EM Bokashi® per tonne of feed + 3% clinoptilolite (group III); basal diet + 0.5% multi-strain probiotic formulation EM Bokashi® per tonne of feed + 3% clinoptilolite + *E. tenella* infection (group IV); basal diet + 0.8% multi-strain probiotic formulation EM Bokashi® per tonne of feed + 3% clinoptilolite and *E. tenella* infection (group V); and basal diet + *E. tenella* infection (group VI). The additives were introduced into the experimental diets in place of wheat. The experimental design and composition of the basal diet are shown in Tables 1 and 2 and in Figure 1. The basal diet was formulated to meet dietary recommendations for Ross 308 broiler chickens [37].

Table 1. Experimental design.

Group	Basal Diet	Addition to Basal Diet		Infection 14 Days of Age
		Multi-Strain Probiotic Formulation EM Bokashi®	Clinoptilolite	
I	+	-	-	-
II	+	0.5%	3%	-
III	+	0.8%	3%	-
IV	+	0.5%	3%	1.7×10^4 *E. tenella* *
V	+	0.8%	3%	1.7×10^4 *E. tenella* *
VI	+	-	-	1.7×10^4 *E. tenella* *

* Number of sporulated oocysts inoculated into the crop.

Table 2. Composition and nutrient value of basal diet (%).

Component	Percentage %		
	Starter (Days 1–21)	**Grower (Days 22–35)**	**Finisher (Days 36–42)**
Wheat (group I, VI)	35.02	40.00	49.73
Wheat (group II, IV)	31.52	36.50	46.23
Wheat (group III, V)	31.22	36.20	45.93
Soybean meal	34.02	29.40	22.88
Maize	22.54	17.21	10.03
Rapeseed oil	2.01	2.50	4.11
Lard	2.00	2.97	3.50
Rapeseed meal	1.00	5.00	7.00
Premix without coccidiostat [1]	1.00	1.00	1.00
Monocalcium phosphate	0.72	0.55	0.41
Calcium carbonate	0.62	0.44	0.26
L-Methionine	0.30	0.24	0.18
L-Lysine	0.22	0.18	0.20
Sodium chloride	0.20	0.20	0.20
$NaHCO_3$	0.12	0.16	0.12
Threonine	0.12	0.08	0.07
L-valine	0.12	0.06	0.03
Optiphos (0.01%) [2]	0.01	0.01	0.01
AMEN	12.47	12.77	13.39
Crude protein	22.7	21.94	20.14
Crude fat	6.02	7.36	9.33
P available	0.48	0.43	0.4
Ca	0.96	0.87	0.79
Na	0.16	0.16	0.16
Cl	0.16	0.16	0.16
Lys dig.	1.25	1.15	1.05
Met dig.	0.6	0.54	0.46
Thr dig.	0.84	0.77	0.7
Val dig	0.94	0.87	0.79

[1] Vitamin–mineral premix provided per kg diet: Mn, 55 mg; Zn, 50 mg; Fe, 80 mg; Cu, 5 mg; Se, 0.1 mg; I, 0.36 mg; Na, 1.6 g, retinol, 2.48 mg; cholecalciferol, 25 µg; DL-α-tocopherol, 60 mg; cyanocobalamin, 0.012 mg; menadione sodium bisulphite, 1.1 mg; niacin, 53 mg; choline chloride, 1020 mg; folic acid, 0.75 mg; biotin, 0.25 mg; riboflavin, 5.5 mg; and xylanase (Econase HCP 4000; AB Vista, Marlborough, UK), 4 mg. [2] Optiphos—6-phytase derived from *E. coli*.

The chickens had unlimited access to feed and water. They received compound feeds appropriate for each rearing period: starter, S (days 1–21); grower, G (days 22–35); and finisher, F (days 36–42). The starter feed was provided to the chickens in crumble form, and the grower and finisher feeds were pelleted. No coccidiostats or antibiotics were used in the experiment.

The rearing period was 42 days. The experimental birds were housed in pens on wood shavings in a room with controlled temperature and humidity. Before the birds were placed in the pens, the wood shavings were tested for the presence of *Eimeria* spp. oocysts, according to Hauck and Pacheco [38], and no *Eimeria* oocysts were found. The pens were equipped with feeding lines and nipple drinkers. The lighting regime was adjusted to the age and diurnal rhythm of the birds. The light intensity was 30–40 lux up to day 7 and 20–30 lux thereafter. Three days before the chickens were placed in the cages, the floor was heated to 29 °C and the air to 33 °C. The temperature was maintained at 31–33 °C up to day 7 and then gradually reduced by 2 °C a week to a final temperature of 22–23 °C. The relative humidity throughout the experiment was 60% +/− 10%. The concentrations of gases were <10 ppm for ammonia and <3000 ppm for carbon dioxide.

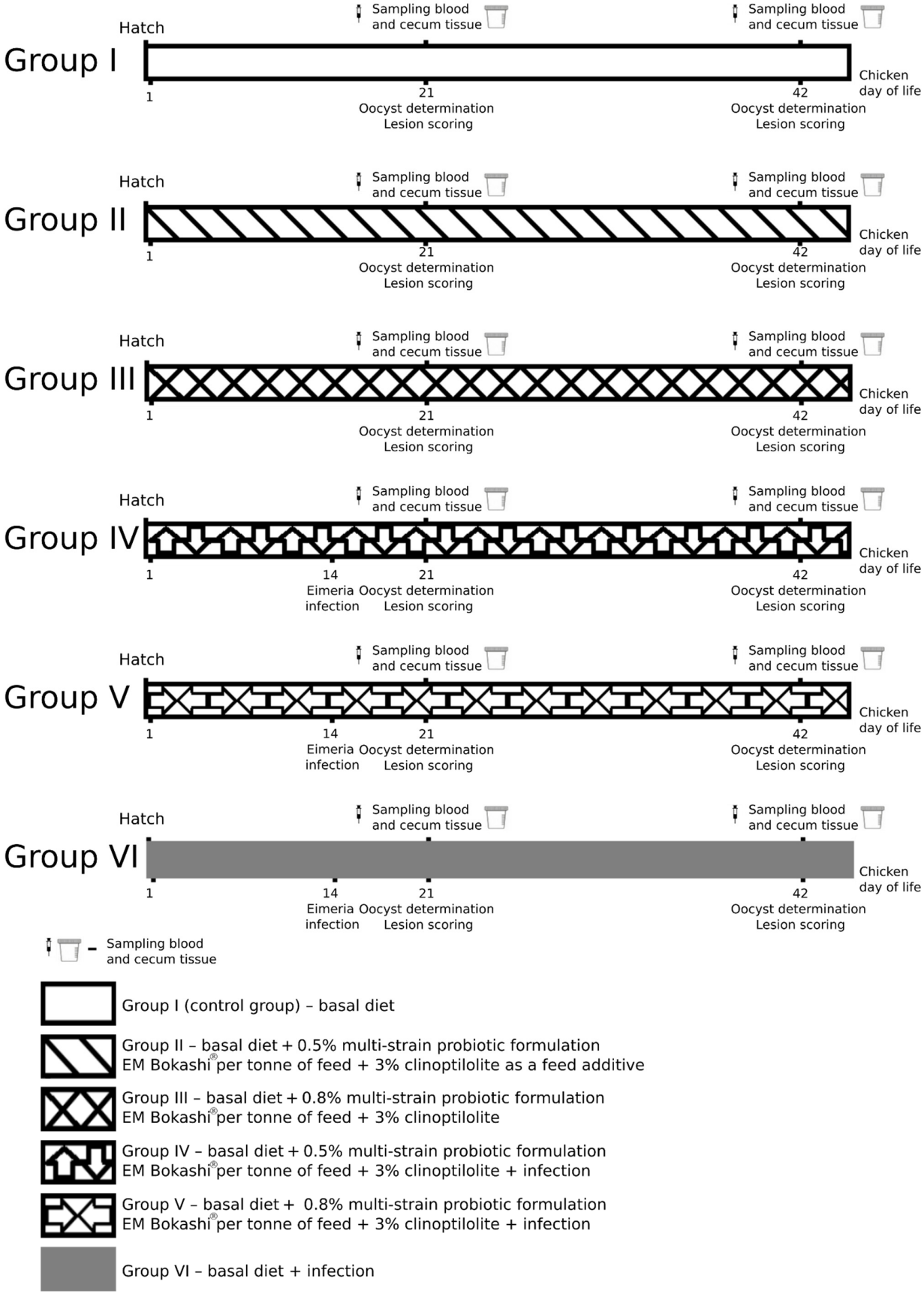

Figure 1. Experimental flowchart.

The multi-strain probiotic formulation EM Bokashi° (batch number 45/01/2020) used in the experiment is manufactured by the commercial company Greenland Technologia EM, Janowiec, Poland, and contains a mixture of microorganisms (Table 3). The manufacturer tested the viability of probiotic bacterial cells and their content per gram of product. The company laboratory operates in compliance with all criteria for food safety and production quality, and the manufacturer has obtained a veterinary approval number for the product (α PL 0614002p, Quality Certificate, Supplementary Materials). Before the start of the experiment, the microbiological purity of the probiotic preparation was tested in the National Reference Laboratory of the Department of Hygiene of Animal Feedingstuffs, National Veterinary Research Institute in Puławy (Certificate of Analysis—Test Report no. P/20/59139, Supplementary Materials). To prevent loss of viability of the microbial strains in the product, the feed for the control and experimental groups was prepared once a week throughout the experiment.

Table 3. Composition of EM Bokashi® composition.

	Microbial Composition	Strain Number	Content per Gram of Product
1.	*Saccharomyces cerevisiae*	Y200007	5×10^4 CFU/g
2.	*Lactobacillus casei*	ATCC 7469	5×10^8 CFU/g
3.	*Lactobacillus plantarum*	ATCC 8014	5×10^8 CFU/g
4.	*Enterococcus faecalis*	UC-100 (CGMCC No.1.0130)	2.5×10^6 CFU/g
5.	*Enterococcus faecium*	NCIMB SF68	5×10^9 CFU/g

In groups II–V, clinoptilolite (Andalusia sp. z o.o., Warsaw, Poland) was added to the feed in the amount of 3%. The preparation contained at least 87% clinoptilolite as the active substance, with the following composition: 67.07% SiO_2, 12.4% Al_2O_3, 2.09% CaO, 2.8% K_2O, 0.9% Fe_2O_3, 0.72% MgO, 2.05% Na_2O, 0.19% TiO_2, 0.04% MnO, and 0.014% P_2O_5.

2.2. Parasites and Inoculum Preparation

A local field isolate of *Eimeria tenella* recovered from a case of caecal coccidiosis in a poultry flock (a flock of 150,000 broiler chickens), whose carcasses were submitted for post-mortem examination at the Department of Avian Diseases, Faculty of Veterinary Medicine, University of Life Sciences in Lublin, was used for the challenge of chickens in groups IV, V, and VI. Oocysts were replicated, isolated, and sporulated using standard procedures described by Raether et al. [39]. Only sporulated oocysts that had been stored for no longer than 4 weeks after their acquisition were used to infect the chickens. The oocysts were confirmed to belong to the species *E. tenella* by PCR using species-specific forward primer 5′- AATTTAGTCCATCGCAACCCTTG -3′ and reverse primer 5′- CGAGCGCTCTGCAT-ACGACA -3′ as described by Lee et al. [40]. All chickens from each replicate in groups IV, V, and VI were infected at 14 days of age with 1.7×10^4 *E. tenella* sporulated oocysts per bird by oral inoculation into the crop [41].

2.3. Clinical Signs and Growth Performance in Chickens

The birds were under clinical observation throughout the experiment, with special attention paid to their activity, appetite, respiratory symptoms, and the occurrence of digestive disorders manifesting as diarrhoea. The health status of the birds was evaluated by determining clinical parameters, anatomopathological changes in dead birds, and the mortality rate (Table 4). During the experiment, the birds were weighed before feeding on days 0, 7, 14, 21 (end of starter period), 28, 35 (end of grower period), and 42 (end of finisher period). In addition, feed intake (FI) and feed conversion ratio (FCR) were recorded on a per-pen basis on days 0, 7, 14, 21, 28, 35, and 42. Finally, adjusted average daily gain (ADG), feed intake (FI), and feed conversion ratio (FCR) were calculated for each period (days 0–21, 22–35, and 36–42) and also for the cumulative experimental period (days 0–42).

The feed conversion ratio (FCR) for all experimental groups during the 42-day period was calculated as mean feed consumption/mean weight. Chicken mortality was recorded daily during morning and afternoon inspections and used for chicken-day calculations. Feed intake and FCR were corrected for mortality accordingly (Table 5).

Table 4. Evaluation of health parameters.

Item	Group I	Group II	Group III	Group IV	Group V	Group VI
Mortality rate (%)	8.00% (8 birds)	6.00% (6 birds)	5.00% (5 birds)	5.00% (5 birds)	4.00% (4 birds)	11.00% (11 birds)
Gastrointestinal symptoms	Diarrhoea lasting 5–6 days and remitting spontaneously (n = 16).	Diarrhoea lasting 2 days and remitting spontaneously (n = 22).	None	Diarrhoea with mucus and blood lasting 6 days and remitting spontaneously (n = 45).	Diarrhoea with mucus and blood lasting 4 days and remitting spontaneously (n = 52).	Diarrhoea with mucus and blood lasting 7 days (n = 18) andbloody diarrhoea (n = 39).
Respiratory symptoms	Cough n = 15 Sneezing n = 23 Conjunctivitis n = 36	- Sneezing n = 25 Conjunctivitis n = 28	Cough n = 15 Sneezing n = 26 Conjunctivitis n = 42	Cough n = 8 Sneezing n = 16 Conjunctivitis n = 31	- Sneezing n = 8 Conjunctivitis n = 11	- Sneezing n = 26 Conjunctivitis n = 38
Anatomopathological changes in dead birds	Intestinal hyperaemia, petechiae in the mucosa of the small intestine, and catarrhal enteritis.	Intestinal hyperaemia petechiae in the mucosa of the small intestine.	Intestinal hyperaemia petechiae in the mucosa of the small intestine.	Intestinal hyperaemia, isolated pinpoint petechiae in the mucosa of the small intestine, and catarrhal enteritis.	Intestinal hyperaemia, isolated pinpoint petechiae in the mucosa of the small intestine, and catarrhal enteritis.	Intestinal hyperaemia, isolated pinpoint petechiae in the mucosa of the small intestine, andhaemorrhagic enteritis.

Group I (control group)—basal diet; group II—basal diet + 0.5% multi-strain probiotic formulation EM Bokashi® per tonne of feed + 3% clinoptilolite as a feed additive; group III—basal diet + 0.8% multi-strain probiotic formulation EM Bokashi® per tonne of feed + 3% clinoptilolite; group IV—basal diet + 0.5% multi-strain probiotic formulation EM Bokashi® per tonne of feed + 3% clinoptilolite + *E. tenella* infection; group V—basal diet + 0.8% multi-strain probiotic formulation EM Bokashi® per tonne of feed + 3% clinoptilolite + *E. tenella* infection; and group VI—basal diet + *E. tenella* infection.

Table 5. Growth parameters.

			Starter (0–10)			Grower (11–24)			Finisher (25–42)			Total (0–42)		
Group	**Addition**	**Infection**	**BWG**	**FI**	**FCR**	**BWG**	**FI**	**FCR**	**BWG**	**FI**	**FCR**	**BWG**	**FI**	**FCR**
I	-	-	206	222	1.08	1170	1546	1.31	1666	2593	1.53	3060 bc	4384	1.43
II	0.5%X1 + 3%X2	-	214	222	1.04	1198	1621	1.33	1764	2689	1.56	3077 bc	4518	1.45
III	0.8%X1 + 3%X2	-	213	229	1.06	1226	1627	1.35	1807	2873	1.50	3291a	4794	1.44
IV	0.5%X1 + 3%X2	+	215	229	1.05	1235	1647	1.34	1787	2808	1.57	3237 a	4684	1.45
V	0.8%X1 + 3%X2	+	211	230	1.09	1192	1606	1.38	1787	2814	1.54	3143 ab	4650	1.45
VI	-	+	202	208	1.03	1163	1502	1.33	1596	2631	1.62	2965 c	4235	1.47
SEM			1.720	2.079	0.006	10.610	13.594	0.006	20.031	24.778	0.010	24.873	0.006	0.222
p value			0.1931	0.014	0.0568	0.2954	0.0119	0.0711	0.0093	0.002	0.0157	0.0005	0.0001	0.6452
			Starter (0–10)			**Grower (11–24)**			**Finisher (25–42)**			**Total (0–42)**		
	Addition	**Infection**	**BWG**	**FI**	**FCR**	**BWG**	**FI**	**FCR**	**BWG**	**FI**	**FCR**	**BWG**	**FI**	**FCR**
			Main effects											
	-		204 b	215 b	1.06	1167	1524 b	1.32 b	1631 b	2612 b	1.57 a	3013	4309 b	1.45
	0.5%X1 + 3%X2		215 a	226 a	1.04	1216	1634 a	1.34 ab	1776 a	2749 a	1.57 a	3157	4601a	1.45
	0.8%X1 + 3%X2		212 ab	229 a	1.07	1209	1617 a	1.36 a	1797 a	2843 a	1.52 b	3217	4722 a	1.45
		-	211	224	1.06	1198	1598	1.33	1746	2718	1.53b	3143	4566	1.44
		+	210	222	1.06	1197	1585	1.35	1723	2751	1.57 a	3115	4523	1.46
			p value											
Addition			0.039	0.0105	0.1721	0.1337	0.0017	0.0153	0.001	0.0004	0.0397	0.0009	<0.0001	0.9778

Table 5. *Cont.*

Infection	0.6695	0.5561	0.9414	0.9457	0.6029	0.2876	0.5356	0.452	0.0197	0.5137	0.5359	0.2244
Addition × Infection	0.7893	0.0663	0.1374	0.3738	0.509	0.666	0.5786	0.242	0.1795	0.0085	0.106	0.3972

X1—multi-strain probiotic formulation EM Bokashi®, X2—clinoptilolite; group I (control group)—basal diet; group II—basal diet + 0.5% multi-strain probiotic formulation EM Bokashi® per tonne of feed + 3% clinoptilolite as a feed additive; group III—basal diet + 0.8% multi-strain probiotic formulation EM Bokashi® per tonne of feed + 3% clinoptilolite; group IV—basal diet + 0.5% multi-strain probiotic formulation EM Bokashi® per tonne of feed + 3% clinoptilolite + *E. tenella* infection; group V—basal diet + 0.8% multi-strain probiotic formulation EM Bokashi® per tonne of feed + 3% clinoptilolite + *E. tenella* infection; and group VI—basal diet + *E. tenella* infection. a, b, c—statistical differences, SEM—standard error of the mean

2.4. Faecal Collection and Counting of Oocysts

The numbers of oocysts per gram (OPG) of faeces were determined in samples collected from each pen and from each group on days 21 and 42 of the experiment. For each pen, fresh excreta samples were collected from every corner of the pen and from the centre of the pen and placed in separate airtight plastic bags. The number of oocysts per gram of faeces was counted using a modified McMaster technique as described by Hodgson [42]. Briefly, a 10% (*w/v*) faeces suspension in a salt solution (151 g NaCl mixed into 1 L of water) was prepared. After thorough shaking to obtain a homogenous mixture, 1 mL of the suspension was mixed with 9 mL of a salt solution (131 g of NaCl mixed into 1 L of water). Then, the suspension was pipetted into a McMaster chamber, which has two chambers with two identical 10 mm × 10 mm × 1.5 mm grids (0.15 mL). The total number of oocysts under both grids was recorded. The oocyst counts (oocysts per gram) were calculated by multiplying the total number of oocysts in the two chambers by 100. The caecum contents (2 g) were collected aseptically post-mortem from all birds on days 21 and 42 of the experiment. The oocyst counts in the caecum contents were determined as described above.

2.5. Lesion Scoring

On days 21 and 42, two birds per replicate (10 birds/treatment) were randomly selected for scoring of coccidian intestinal lesions. The 0–4 lesion scoring system of Johnson and Reid [43] was used. The areas scored were the duodenum, jejunum, ileum, and caecum. Based on the severity of the lesions, a score of 0 (no lesions), 1 (mild lesions), 2 (moderate lesions), 3 (severe lesions), or 4 (extremely severe lesions) was recorded for each chicken. Dead birds were scored as 4 [43]. Based on the anatomopathological changes observed in the internal organs of the dead birds, the cause of their deaths was established as coccidiosis.

2.6. Gastrointestinal Permeability

Fluorescein isothiocyanate dextran (FITC-d; MW 4000; Sigma-Aldrich, Poznań, Poland) was administered to evaluate gastrointestinal permeability. On days 21 and 42 of the experiment, 10 chicks from each group (two chickens selected randomly from each replicate) were gavaged with 1 mL of FITC-d solution (2.2 mg/mL) according to the method described by Kuttappan et al. [44]. At 2 h post-inoculation, peripheral blood was collected from the wing vein. Blood samples were stored in a dark container for an additional 2 h at room temperature before centrifugation at 1000× *g* for 15 min. A 100 µL volume of serum was transferred from the blood sampling tubes to a dark 96-well microplate. Five levels of FITC-d standard solution were formulated with the stock solution (2.2 mg/mL) and pooled serum from 10 additional unchallenged chickens raised in the same house. The FITC-d levels in the serum samples and standard solution were measured at an excitation wavelength of 485 nm and an emission wavelength of 528 nm using a microplate reader (Spectramax M5, Molecular Devices, San Jose, CA, USA). Blood processing and preparation of the standard solution were performed in a dark environment to protect the FITC-d from light exposure.

2.7. Intestinal Sample Collection

On day 21, 10 chicks from each group (two chickens selected randomly from each replicate) were sacrificed for tissue sampling. The remaining chicks continued to receive experimental diets up to day 42 of the experiment. On day 42, 10 birds (two chickens selected randomly from each replicate) from all groups were sacrificed for tissue sampling. The tissue was sampled from approximately the same central part of the caecum and flushed with cold PBS. Slices were stored at −80 °C until gene expression analysis. The intestinal samples were not pooled.

2.8. Quantitative Real-Time PCR for Tight Junction Proteins, Mucin and Antioxidant Gene Expression

Total RNA was isolated from homogenised samples of the caecum of each bird using the RNeasy Mini Kit (QIAGEN, Crawley, United Kingdom) following the manufacturer's instructions. Purified RNA was eluted in 50 µL RNase-free water and stored at −80 °C until use. RNA was quantified using an ND-1000 spectrophotometer at 260 nm/280 nm (NanoDrop Technologies, Silverside, Wilmington, NC, USA). cDNA for quantitative reverse transcription-PCR (qRT-PCR) was synthesised from 1 µg of purified RNA using 50 ng of random hexamers and the SuperScript II first-strand cDNA synthesis kit according to the manufacturer's instructions (Invitrogen, Chorzów, Poland). The mRNA expression of tight junction proteins, mucin, and antioxidants was determined by qRT-PCR using the Applied BioSystems 7500 real-time PCR system (Applied Biosystems, Warrington, United Kingdom) as previously described by Lammers et al. [45]. The PCR conditions were denaturation at 95 °C for 10 min followed by amplification at 60 °C for 1 min for 40 cycles. The primer sequences used for qRT-PCR are listed in Table 6. Each sample was subjected to qRT-PCR in triplicate, and the mean values were used for analysis. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the reference gene for gene expression. The threshold cycle (C_T) values for the genes of interest were normalised to the average C_T value of the housekeeping genes, and the relative expression of each replicate was calculated as $2^{-\Delta\Delta Ct}$ (Applied Biosystems user Bulletin #2; AI prism 7700 detection system, 2001). The C_T values of each gene were normalised against reference genes.

Table 6. Primers used for quantification of mRNA expression of tight junction proteins, mucin, and antioxidant genes by qRT-PCR.

RNA Target	Primer	Sequence (5′→3′)	Source of Reference	Accession No.
CLDN1	For Rev	5′- TGGAGGATGACCAGGTGAAGA -3′ 5′- CGAGCCACTCTGTTGCCATA -3′	Teng et al., 2021	NM_001013611.2
CLDN2	For Rev	5′- CCTGCTCACCCTCATTGGAG -3′ 5′- GCTGAACTCACTCTTGGGCT -3′	Teng et al., 2021	NM_001277622.1
OCLDN	For Rev	5′- ACGGCAGCACCTACCTCAA -3′ 5′- GGCGAAGAAGCAGATGAG -3′	Teng et al., 2021	NM_205128.1
ZO1	For Rev	5′- CAACTGGTGTGGGTTTCTGAA -3′ 5′- TCACTACCAGGAGCTGAGAGGTAA -3′	Teng et al., 2021	XM_015278981.2
ZO2	For Rev	5′- ATCCAAGAAGGCACCTCAGC -3′ 5′- CATCCTCCCGAACAATGC -3′	Teng et al., 2021	NM_204918.1
JAM2	For Rev	5′- AGCCTCAAATGGGATTGGATT -3′ 5′- CATCAACTTGCATTCGCTTCA -3′	Teng et al., 2021	NM_001006257.1
MUC2	For Rev	5′- ATGCGATGTTAACACAGGACTC -3′ 5′- GTGGAGCACAGCAGACTTTG -3′	Teng et al., 2021	JX284122.1
SOD1	For Rev	5′- ATTACCGGCTTGTCTGATGG -3′ 5′- CCTCCCTTTGCAGTCACATT -3′	Wickramasuriya et al., 2021	NM205064.1

Table 6. *Cont.*

RNA Target	Primer	Sequence (5′→3′)	Source of Reference	Accession No.
CAT	For Rev	5′- ACTGCAAGGCGAAAGTGTTT -3′ 5′- GGCTATGGATGAAGGATGGA -3′	Wickramasuriya et al., 2021	NM001031215.1
HMOX1	For Rev	5′- CTGGAGAAGGGTTGGCTTTCT -3′ 5′- GAAGCTCTGCCTTTGGCTGTA -3′	Wickramasuriya et al., 2021	NM205344
GAPDH [a]	For Rev	5′- CCTCTCTGGCAAAGTCCAAG -3′ 5′- GGTCACGCTCCTGGAAGATA -3′	Teng et al., 2021	NM_204305.1

GAPDH—glyceraldehyde-3-phosphate dehydrogenase; CLDN1—claudin 1; CLDN2—claudin 2; OCLDN—occludin; ZO1—zonula occludens 1; ZO2—zonula occludens 2; JAM2—junctional adhesion molecule 2; MUC2—mucin; HMOX1—haem oxygenase 1; SOD1—superoxide dismutase 1; CAT—catalase; F—forward primer; R—reverse primer; and [a] housekeeping gene.

2.9. Statistical Analysis

Statistical analysis of the results for LS (lesion scoring), OPG (oocysts per gram), and protein expression for the six experimental groups on the two testing days (days 21 and 42) was performed using Statistica 13.2 PL software (StatSoft, Krakow, Poland). Due to the qualitative nature of the lesion score (LS) data (0–4), we used a non-parametric Kruskal–Wallis one-way ANOVA on ranks with multiple comparisons and the Mann–Whitney U test to show statistically significant differences between groups on the two testing days. The significance of differences in the numbers of oocysts (OPG) and expression of proteins, due to the lack of normal distribution of the data, was analysed by a non-parametric Kruskal–Wallis one-way ANOVA on ranks with multiple comparisons. The results were presented in graphic form using the mean and standard deviation (SD), with the same letters indicating the absence of statistically significant differences for $p < 0.05$.

The statistical evaluation of performance results was carried out using SAS® v. 9.4 statistics software (SAS, 2011). All data were presented as mean values with pooled standard error of the mean (SE). A two-way ANOVA was used to determine the effect of the experimental factors. For all characteristics, the significance of differences between group mean values was verified by Tukey's test.

3. Results

3.1. Clinical Signs and Growth Performance in Chickens

During the experiment, the highest percentage of mortality rate, 11%, was observed in the group of birds receiving the basal diet and infected with *E. tenella* (group VI). Symptoms of diarrhoea with mucus and blood lasting 4 to 6 days were observed in birds from all groups infected with *E. tenella*. Intestinal hyperaemia, isolated pinpoint petechiae in the small intestinal mucosa, and catarrhal enteritis were observed among the anatomopathological changes in dead birds in groups infected with *E. tenella* and simultaneously supplemented with probiotic and clinoptilolite (groups IV and V). Additionally, haemorrhagic enteritis was observed in the group receiving the basal diet and infected with *E. tenella.* Detailed data are presented in Table 4 and Figure 2.

The effect of experimental factors on broiler chicken performance is presented in Table 5. During the first 10 days of the experiment, the infection did not affect the performance of broiler chickens ($p > 0.05$). However, the use of the multi-strain probiotic formulation and clinoptilolite statistically significantly increased BWG and FI ($p < 0.05$). Irrespective of the dose of the probiotic, broilers fed supplemented diets had increased FI and poorer FCR ($p < 0.05$). In the final periods of the experiment (days 25–42), infection adversely affected FCR, but the higher dose of the multi-strain probiotic formulation and clinoptilolite statistically significantly decreased the value of this parameter. At the same time, the supplement increased FI but also significantly increased BWG ($p < 0.05$). No interactions between the experimental factors were confirmed during the finisher period ($p > 0.05$). Analysis of the entire experimental period (days 0–42) revealed an interaction between the use of the supplement and infection with BWG ($p < 0.05$). This study showed

that the multi-strain probiotic formulation and clinoptilolite were more effective in infected birds. In the case of other parameters (FI and FCR), the supplement increased FI ($p < 0.05$) but did not affect FCR ($p > 0.05$).

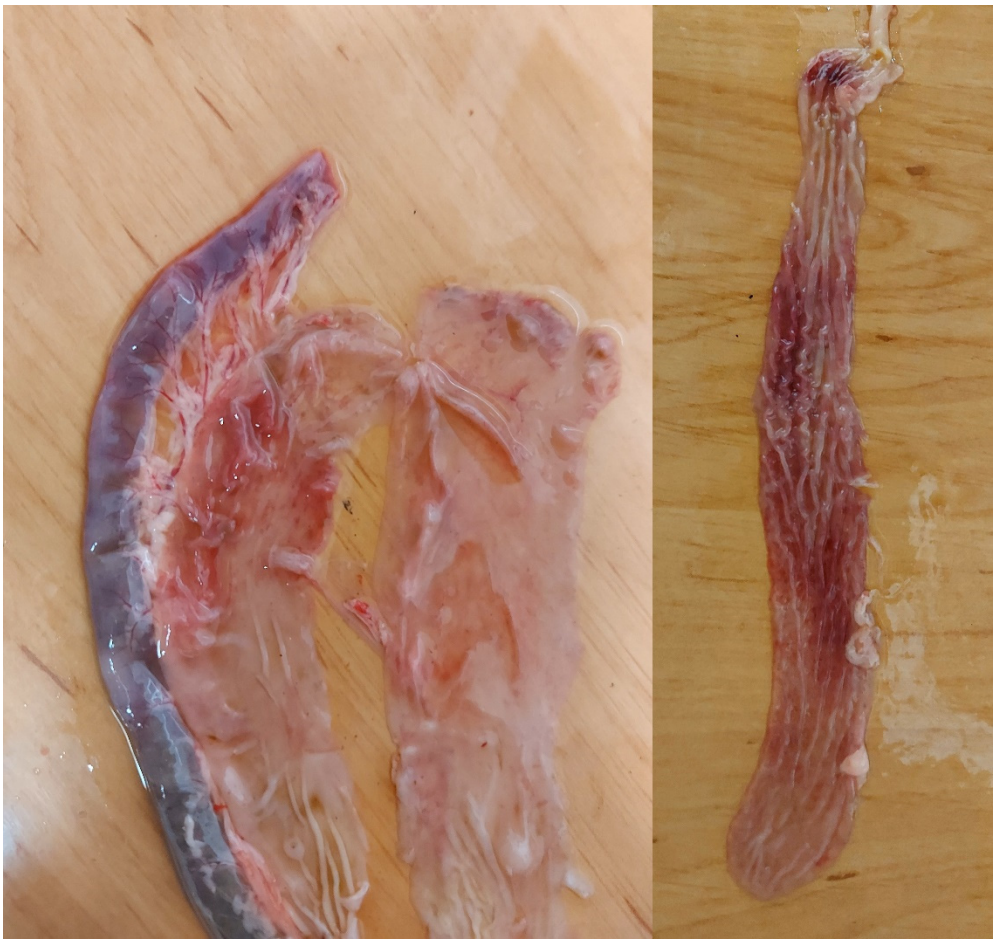

Figure 2. The pathological findings in ceca of *E. tenella* infected broiler chickens. Gross appearance of ceca showing thickening of the intestinal wall and congestion.

3.2. Numbers of Oocysts per Gram of Faeces

Analysis of the mean number of oocysts in the faeces showed that the testing day was statistically significant only for group V (0.8% probiotic + clinoptilolite, infected with *E. tenella*) and group VI (infected with *E. tenella*). The number of oocysts in the faeces of birds in group V was significantly higher ($p \leq 0.05$) on the 21st day of this study and decreased significantly ($p \leq 0.05$) on day 42. In group VI, the number of oocysts in both periods was the highest among all groups and significantly increased ($p \leq 0.05$) on day 42. The number of oocysts in group I (control) was significantly lower ($p \leq 0.05$) than in groups II and V. The mean number of oocysts per gram of faeces in the control group was slightly lower than in group II and slightly higher than in groups III and V. In group VI (infected with *E. tenella*), there was a pronounced increase in the number of oocysts compared to the other groups of chickens (Figure 3).

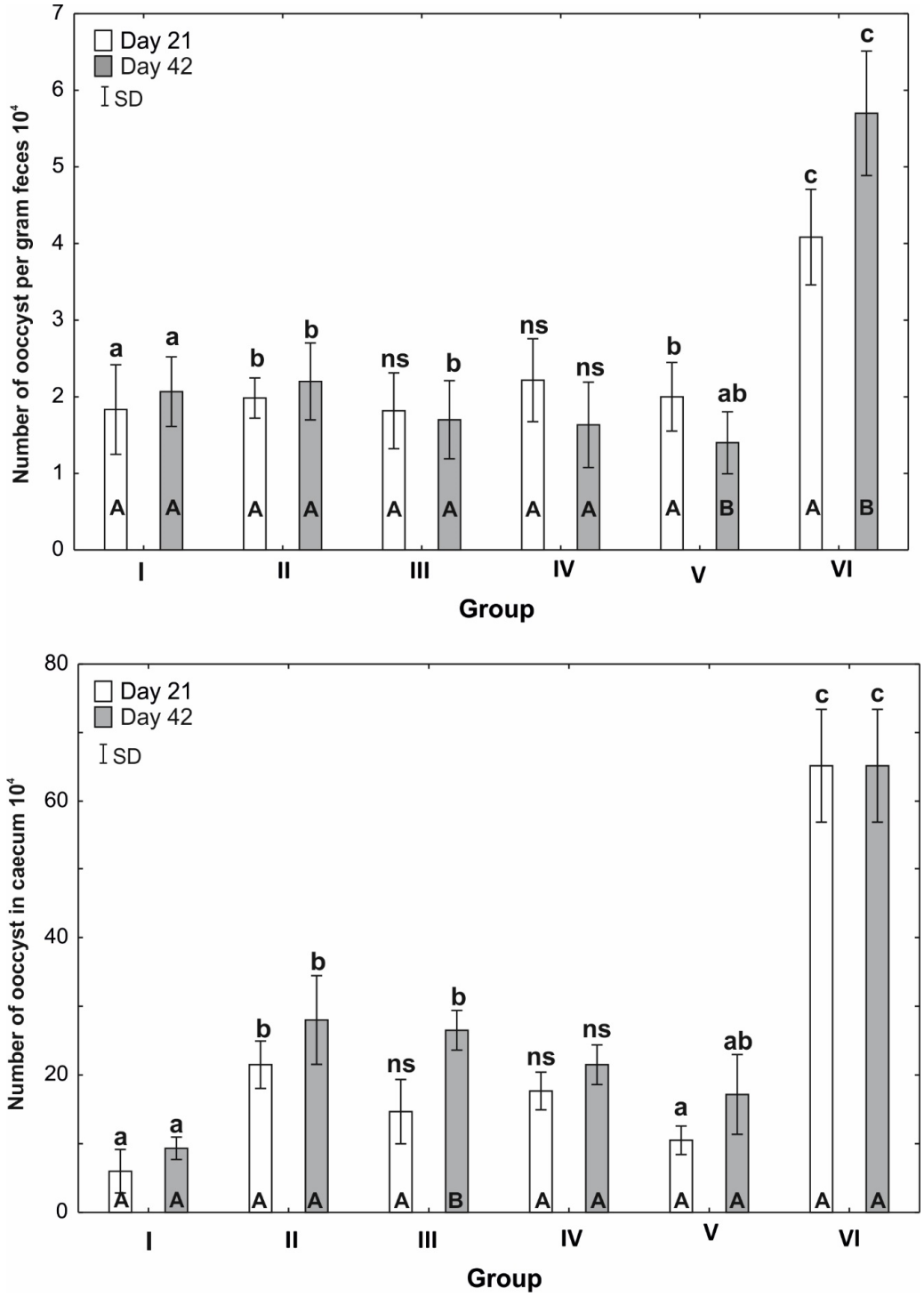

Figure 3. Results of the Kruskal–Wallis one-way ANOVA test (lowercase letters—a, b, c) and the Mann–Whitney U test (uppercase letters—A, B) for the number of oocysts per gram of faeces and the number of oocysts in the caecum of chickens at 21 and 42 days of age. Statistical differences ($p \leq 0.05$) are marked with different letters; ns—statistically non-significant differences. Group I (control group)—basal diet; group II—basal diet + 0.5% multi-strain probiotic formulation EM Bokashi® per tonne of feed + 3% clinoptilolite as a feed additive; group III—basal diet + 0.8% multi-strain probiotic formulation EM Bokashi® per tonne of feed + 3% clinoptilolite; group IV—basal diet + 0.5% multi-strain probiotic formulation EM Bokashi® per tonne of feed + 3% clinoptilolite + *E. tenella* infection;group V—basal diet + 0.8% multi-strain probiotic formulation EM Bokashi® per tonne of feed + 3% clinoptilolite + *E. tenella* infection; and group VI—basal diet + *E. tenella* infection.

3.3. Numbers of oocysts in the Caecum

A detailed analysis of the mean number of oocysts in the caecum showed that the measurement day was a significant factor only for group III (0.8% Bokashi® formulation + 3 clinoptilolite), in which the number of oocysts on day 21 of this study was statistically significantly lower ($p \leq 0.05$) than on day 42. The number of oocysts in the caecum was the lowest in groups I (control) and V and was significantly different from that observed in groups II and VI. Similarly, on day 42 of this study, the control group and group V had the lowest number of oocysts, which significantly differed from the number of oocysts recorded in groups II and III. In group VI (infected with *E. tenella*), the number of oocysts tripled to over 60 (Figure 3).

3.4. Scoring of Coccidial Intestinal Lesions

On day 21 of this study, the lesion score (LS) for the caecum and rectum in group V was statistically significantly lower ($p \leq 0.05$) than in group VI (with the highest LS value, $p = 0.0042$). On day 42, differences in LS values were also shown only for the caecum and rectum; in groups III and V, the LS was statistically significantly lower ($p \leq 0.05$) than in group VI (infected with *E. tenella*).

The results of the Kruskal–Wallis ANOVA for individual intestinal sections on day 21 of this study showed that the mean LS for the duodenum and jejunum was statistically significantly lower ($p \leq 0.05$) than for the caecum and rectum in groups I and VI. On day 42 of this study, the LS was lower for the duodenum than for the caecum and rectum in groups II, III, and VI. There were significant differences in lesion scores between the upper and middle intestines and the lower intestine in groups I–V. In groups I, III, IV, and V, the LS did not differ significantly for the duodenum, jejunum, and ileum, while clear differences were found for the caecum and rectum. In group II, statistically significant differences ($p \leq 0.05$) were found between the ileum and the duodenum and jejunum and between the caecum and rectum and the other sections of the intestines (Table 7).

Table 7. Lesion scoring in chicken intestine.

	Part of Intestine							
	Duodenum (I)		**Jejunum (II)**		**Ileum (III)**		**Ceca and Rectum (IV)**	
	Day of Life							
Group	21	42	21	42	21	42	21	42
I	0.17 ±0.41 A	0.33 ±0.52	0.50 ±0.55	0.67 ±0.52	0.33 ±0.52	1.83 ±0.75	1.00 ±0.63 B	2.17 ±0.75
II	0.33 ±0.52	0.33 ±0.52 A	0.50 ±0.84	0.50 ±0.84	1.17 ±0.75	1.17 ±0.75	1.67 ±0.52	1.67 ±0.52 B
III	0.17 ±0.41	0.17 ±0.41 A	0.33 ±0.52	0.33 ±0.52	0.83 ±0.41	0.83 ±0.41	1.00 ±0.63	1.00 ±0.63 Ba
IV	0.33 ±0.52	0.33±0.52 a	0.17 ±0.41 A	0.17 ±0.41	1.17 ±0.75	1.17 ±0.75	1.50 ±0.55 B	1.50 ±0.55 Ba
V	0.17 ±0.41	0.17 ±0.41	0.17 ±0.41	0.17 ±0.41	0.83 ±0.75	0.83 ±0.75	0.83 ±0.75 a	0.83 ±0.75 b
VI	0.33 ±0.52 A	0.17 ±0.41 A	0.67 ±0.82 A	0.33 ±0.52 A	1.50 ±0.55	2.00 ±0.63	2.67 ±0.82 Bb	3.00 ±0.63 B

a, b—statistical differences ($p \leq 0.05$) in groups of birds. A, B—statistical differences ($p \leq 0.05$) for individual sections of the intestine. Group I (control group)—basal diet; group II—basal diet + 0.5% multi-strain probiotic formulation EM Bokashi® per tonne of feed + 3% clinoptilolite as a feed additive; group III—basal diet + 0.8% multi-strain probiotic formulation EM Bokashi® per tonne of feed + 3% clinoptilolite; group IV—basal diet + 0.5% multi-strain probiotic formulation EM Bokashi® per tonne of feed + 3% clinoptilolite + *E. tenella* infection; group V—basal diet + 0.8% multi-strain probiotic formulation EM Bokashi® per tonne of feed + 3% clinoptilolite + *E. tenella* infection; and group VI—basal diet + *E. tenella* infection.

3.5. Evaluation of Gastrointestinal Permeability

The highest level of FITC-d was observed on day 21 of this study in the group receiving the basal diet and infected with *E. tenella* (group VI) compared to the other groups. Similar results were seen on day 42. Increased FITC recovery on study day 42 was observed in the *E. tenella*-infected groups (IV, V, and VI) compared to sera from the control birds and the groups supplemented but not infected with *E. tenella.* Moreover, the FITC-d level in the sera of birds from groups IV and V on day 42 of the experiment was lower than on day 21. Conversely, in birds from group VI, the FITC-d level in the serum on day 42 of this study was significantly higher ($p \leq 0.05$) than on day 21 (Figure 4).

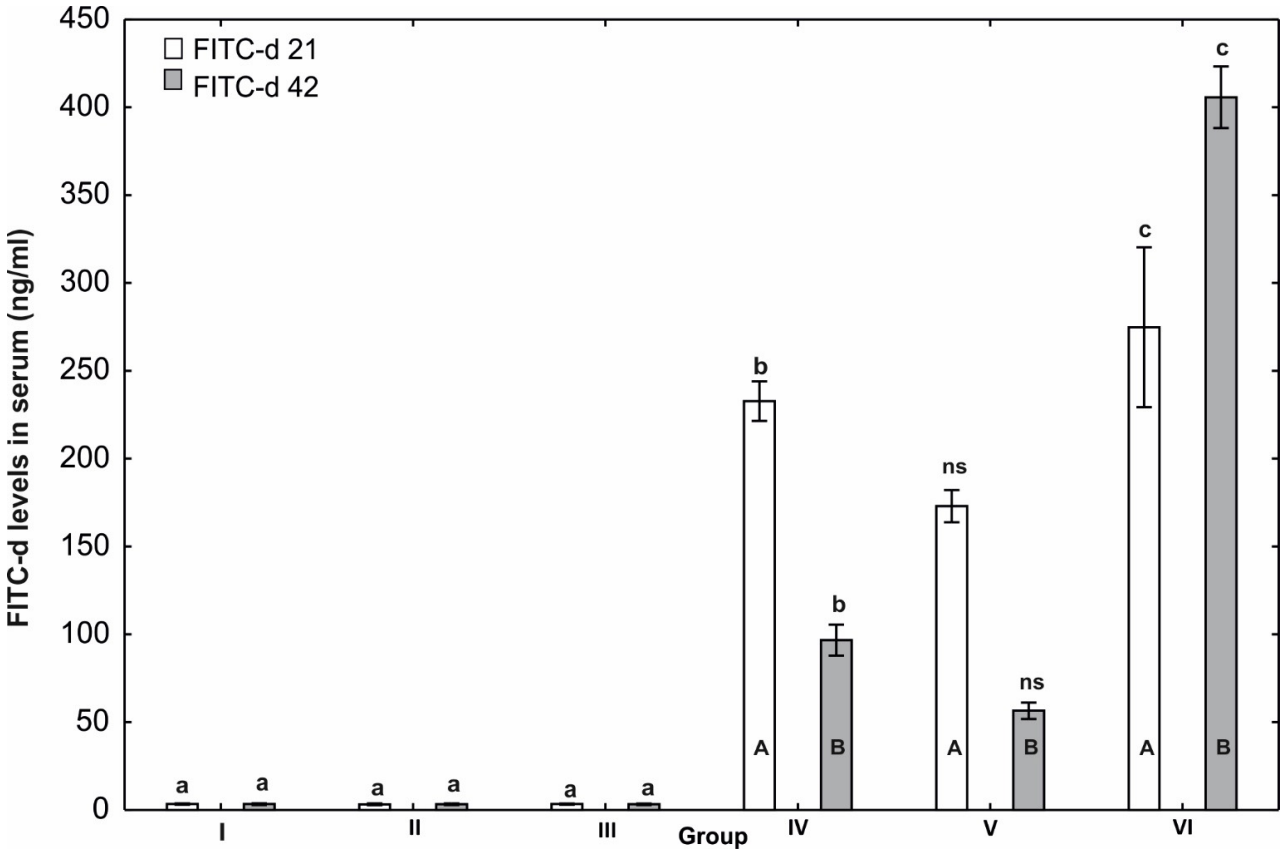

Figure 4. Results of the Kruskal–Wallis one-way ANOVA test (lowercase letters—a, b, c) and the Mann–Whitney U test (uppercase letters—A, B) for FITC-d (fluorescein isothiocyanate dextran) levels in the serum (ng/mL) of chickens at 21 and 42 days of age. Statistical differences ($p \leq 0.05$) are marked with different letters; ns—statistically non-significant differences. Group I (control group)—basal diet; group II—basal diet + 0.5% multi-strain probiotic formulation EM Bokashi® per tonne of feed + 3% clinoptilolite as a feed additive; group III—basal diet + 0.8% multi-strain probiotic formulation EM Bokashi® per tonne of feed + 3% clinoptilolite; group IV—basal diet + 0.5% multi-strain probiotic formulation EM Bokashi® per tonne of feed + 3% clinoptilolite + *E. tenella* infection; group V—basal diet + 0.8% multi-strain probiotic formulation EM Bokashi® per tonne of feed + 3% clinoptilolite + *E. tenella* infection; and group VI—basal diet + *E. tenella* infection.

3.6. mRNA Expression of OCLDN, CLDN1, CLDN2, MUC2, ZO1, ZO2, JAM2, CAT, SOD1 and HMOX1

A comparison of the mRNA expression of OCLDN between the groups on day 21 of this study showed the highest levels of expression in the control group and group VI (1.0 and over 1.2, respectively).

In groups IV, V, and VI, the level of OCLDN expression was higher on day 21 of this study, while in group II it was significantly lower ($p \leq 0.05$) on day 21 than on day 42 (Figure 5).

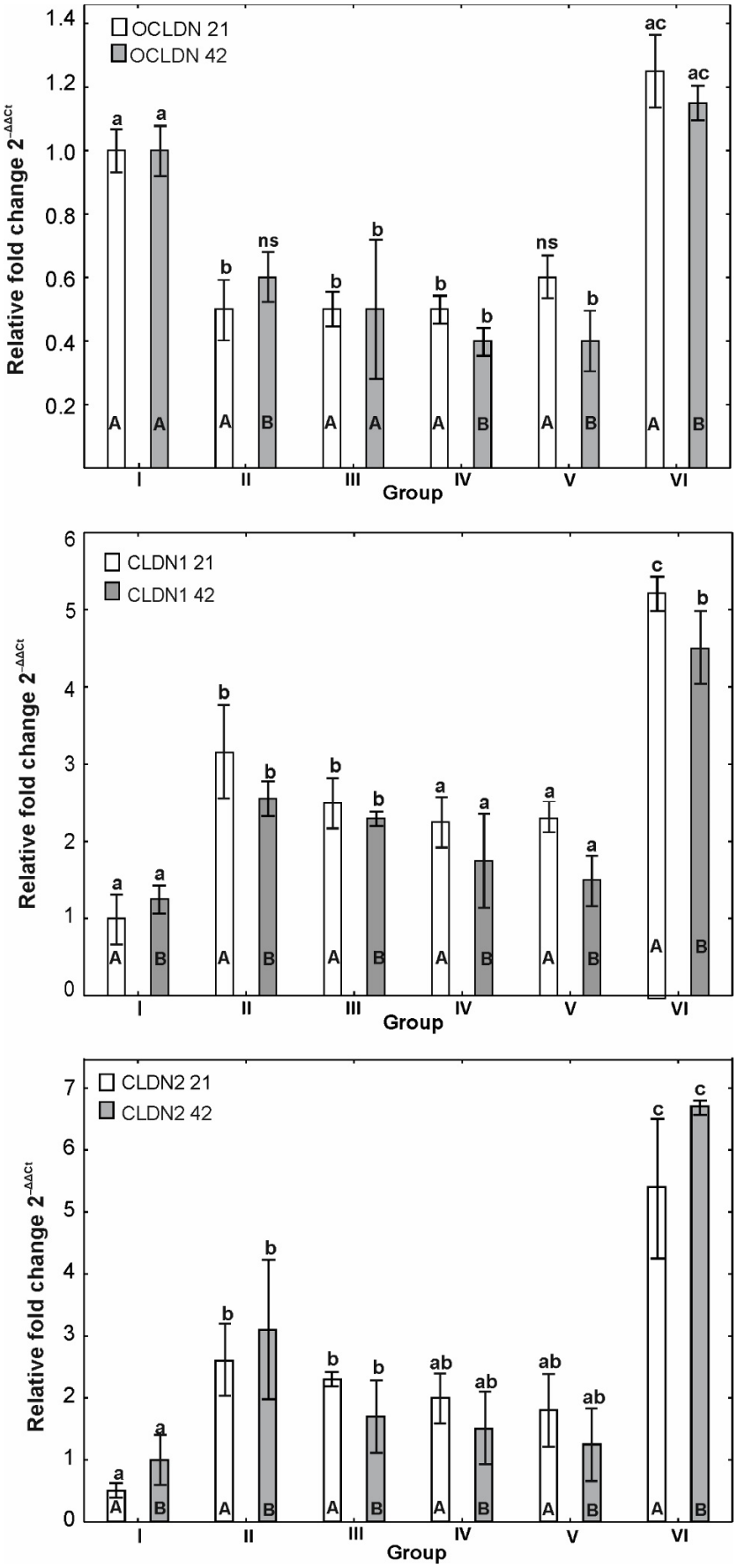

Figure 5. Results of the Kruskal–Wallis one-way ANOVA test (lowercase letters—a, b, c) and the Mann–

Whitney U test (uppercase letters—A, B) for 2 OCLDN—occludin, CLDN1—claudin 1 and CLDN2—claudin 2 gene expression in the chicken intestine (caecum) at 21 and 42 days of age. Statistical differences ($p \leq 0.05$) are marked with different letters; ns—statistically non-significant differences. Group I (control group)—basal diet; group II—basal diet + 0.5% multi-strain probiotic formulation EM Bokashi® per tonne of feed + 3% clinoptilolite as a feed additive; group III—basal diet + 0.8% multi-strain probiotic formulation EM Bokashi® per tonne of feed + 3% clinoptilolite; group IV—basal diet + 0.5% multi-strain probiotic formulation EM Bokashi® per tonne of feed + 3% clinoptilolite + *E. tenella* infection; group V—basal diet + 0.8% multi-strain probiotic formulation EM Bokashi® per tonne of feed + 3% clinoptilolite + *E. tenella* infection; and group VI—basal diet + *E. tenella* infection.

Higher mRNA expression of CLDN1 was observed on the 21st day of this study compared to day 42 in all groups except the control group. The analysis of the mRNA expression of CLDN1 on day 21 of this study showed statistically significant differences ($p \leq 0.05$) between groups IV and V and groups II and III. CLDN1 expression was highest in group VI (infected with *E. tenella*), at 5.25, and differed significantly from the other groups (Figure 5).

Analysis of CLDN2 expression on the 21st day of this study showed statistically significantly higher ($p \leq 0.05$) expression of CLDN2 in groups II, III, and VI compared to the control group. In group VI (infected with *E. tenella*), the expression of CLDN2 was statistically significantly higher ($p \leq 0.05$) than in the control group and other experimental groups on both days of this study (Figure 5).

Statistically significant differences ($p \leq 0.05$) in the level of MUC2 expression, depending on the measurement day, were observed for groups IV and V, in which it was more than twice as high on day 42 as on day 21. Comparison of MUC2 expression between groups on day 21 of this study showed that it was statistically significantly ($p \leq 0.05$) higher in the control group. On day 42 of this study, the mRNA expression level of MUC2 was the highest in group V and the lowest in group VI (Figure 6).

The level of mRNA of ZO1 expression on the 21st day of the experiment was highest in the control group and differed significantly from the levels in groups II, III, and VI. The level of this protein expression on the 42nd day of this study in the control group was statistically significantly higher ($p \leq 0.05$) than in groups II, III, IV, and VI (Figure 6).

A comparison of the ZO2 expression level between groups on day 21 showed that it was statistically significantly higher ($p \leq 0.05$) in the control group than in groups II, III, and IV but did not differ significantly from the level in groups V and VI. On day 42, the ZO2 expression level was higher in groups I and V than in groups II and VI. The differences obtained in groups III and IV were not statistically significant (Figure 6).

JAM2 expression on the 21st day of the experiment was statistically significantly higher ($p \leq 0.05$) in groups II and VI than in group V. The expression of this protein on the 42nd day of the experiment was the highest in group VI and statistically significantly higher ($p \leq 0.05$) in groups II and III than in groups I, IV, and V (Figure 6).

The level of CAT expression on the 21st day of this study in group V was statistically significantly higher ($p \leq 0.05$) than in the other groups. Similarly, on the 42nd day of this study, the level of CAT expression in group VI (infected with *E. tenella*) was statistically significantly lower ($p \leq 0.05$) than in the other groups. In experimental groups II, III, IV, and V and in the control group, a statistically significant increase ($p \leq 0.05$) in CAT expression was observed between days 21 and 42 of this study. In contrast, in group VI, the expression of mRNA of this protein was statistically significantly lower ($p \leq 0.05$) on day 42 of the experiment than on day 21 (Figure 7).

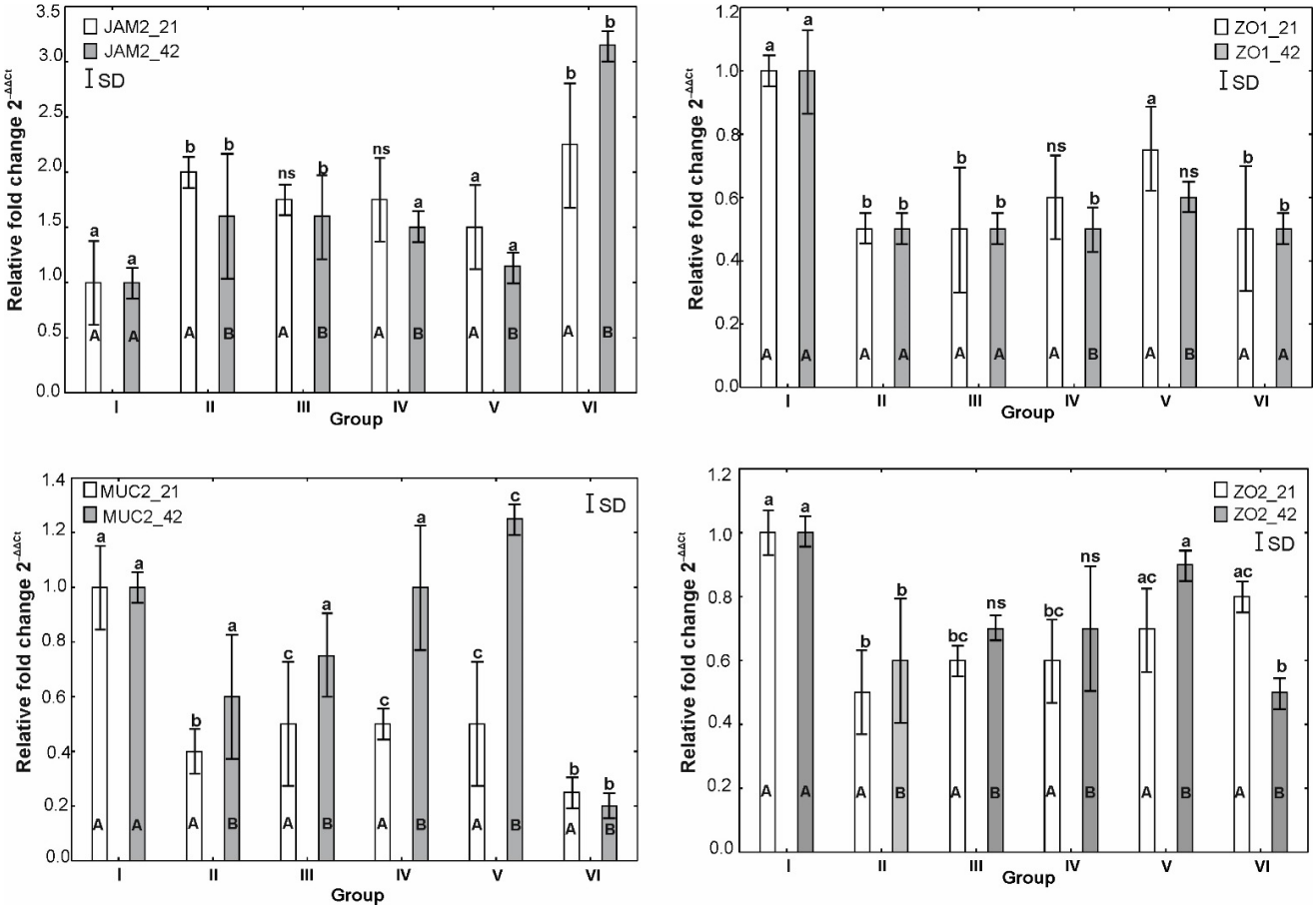

Figure 6. Results of the Kruskal–Wallis one-way ANOVA test (lowercase letters—a, b, c) and the Mann–Whitney U test (uppercase letters—A, B) for 3 JAM2—junctional adhesion molecule 2, MUC2—mucin, ZO1– zonula occludens 1, and ZO2—zonula occludens 2 gene expression in the caecum of chickens at 21 and 42 days of age. Statistical differences ($p \leq 0.05$) are marked with different letters; ns—statistically non–significant differences. Group I (control group)—basal diet; group II—basal diet + 0.5% multi-strain probiotic formulation EM Bokashi® per tonne of feed + 3% clinoptilolite as a feed additive; group III—basal diet + 0.8% multi-strain probiotic formulation EM Bokashi® per tonne of feed + 3% clinoptilolite; group IV—basal diet + 0.5% multi-strain probiotic formulation EM Bokashi® per tonne of feed + 3% clinoptilolite + *E. tenella* infection; group V—basal diet + 0.8% multi-strain probiotic formulation EM Bokashi® per tonne of feed + 3% clinoptilolite + *E. tenella* infection; and group VI—basal diet + *E. tenella* infection.

The expression of mRNA of SOD1 on the 21st day of the experiment was highest in group VI (infected with *E. tenella*). The results obtained for groups III and IV did not differ significantly from the other groups (Figure 7).

The comparison of HMOX1 expression between groups on day 21 of the experiment showed statistically significant values in groups III, IV, and V compared to groups II and VI. Similar results were obtained on day 42, but the level of HMOX1 expression in groups III, IV, and V was higher than on the first day of this study. HMOX1 expression in groups I, II, and VI were statistically significantly lower ($p \leq 0.05$) than in the other groups (Figure 7).

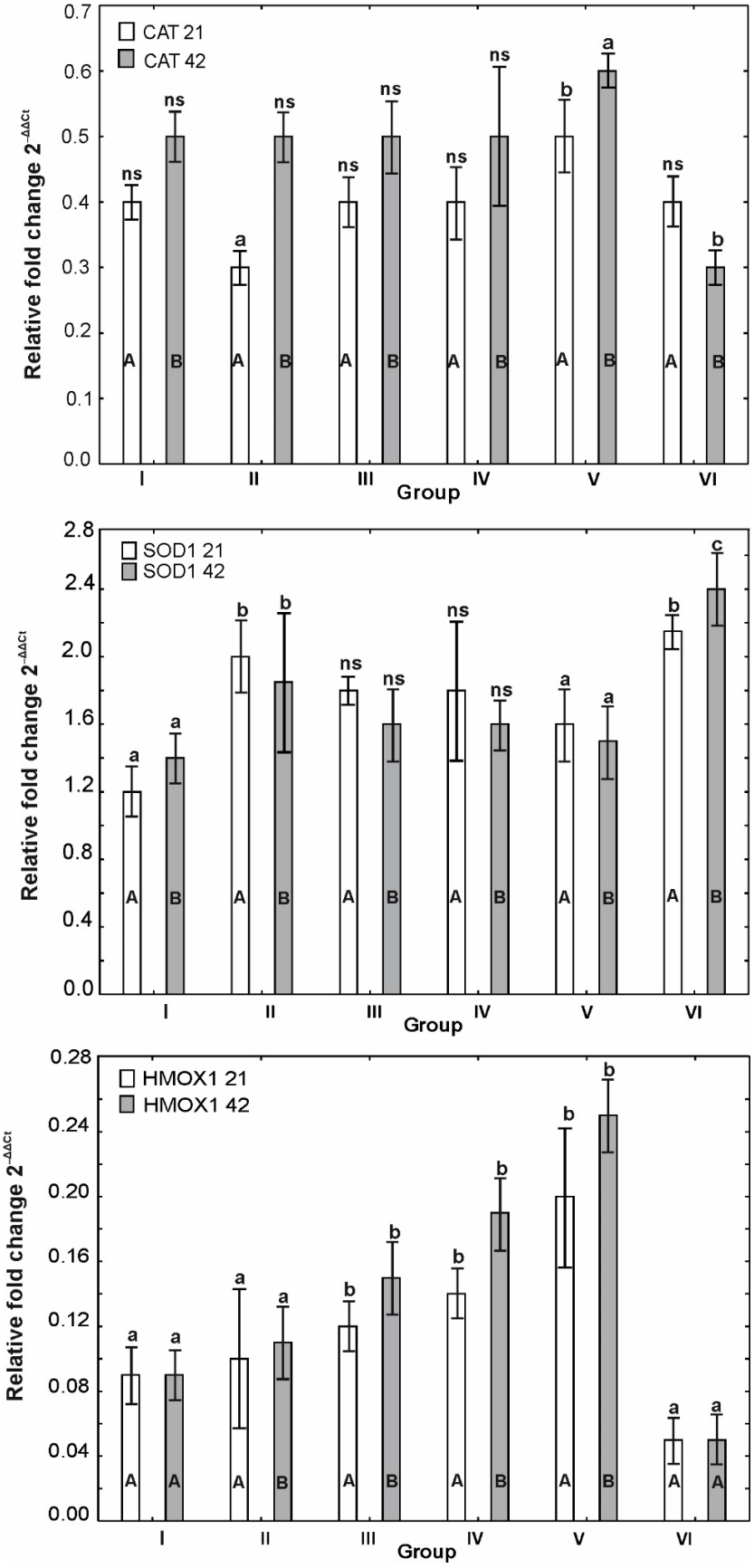

Figure 7. Results of the Kruskal–Wallis one-way ANOVA test (lowercase letters—a, b, c) and the Mann–Whitney U test (uppercase letters—A, B) for CAT—catalase, SOD1—superoxide dismutase 1,

and HMOX1—haem oxygenase 1 gene expression in the caecum of chickens at 21 and 42 days of age. Statistical differences ($p \leq 0.05$) are marked with different letters; ns—statistically non-significant differences. Group I (control group)—basal diet; group II—basal diet + 0.5% multi-strain probiotic formulation EM Bokashi® per tonne of feed + 3% clinoptilolite as a feed additive; group III—basal diet + 0.8% multi-strain probiotic formulation EM Bokashi® per tonne of feed + 3% clinoptilolite; group IV—basal diet + 0.5% multi-strain probiotic formulation EM Bokashi® per tonne of feed + 3% clinoptilolite + *E. tenella* infection; group V—basal diet + 0.8% multi-strain probiotic formulation EM Bokashi® per tonne of feed + 3% clinoptilolite + *E. tenella* infection; and group VI—basal diet + *E. tenella* infection.

4. Discussion

Maintenance of the integrity of the intestinal barrier is dependent on tight connections between intestinal epithelial cells [46–48]. This involves structures of tight junctions (TJ) composed of multiprotein complexes of transmembrane proteins, claudins and occludins, adhesion proteins (JAM—junctional adhesion molecules), tricellulin, and zonulin [49,50]. Malfunctioning of the barrier of the intestinal epithelium and the associated increase in intestinal permeability predispose the birds to the development of numerous diseases with symptoms of gastroenteritis, accompanied by malabsorption and decreased weight gains [49–51].

In the experiment, the integrity of the intestinal barrier was tested in vivo by determining the concentration of fluorescein isothiocyanate dextran (FITC-d) in the serum of chickens following oral administration of FITC-d. During the experiment, damage to the tight junctions between enterocytes was demonstrated in a group of birds aged 21 and 42 days infected with *E. tenella*, which increased the permeability of the intestinal barrier. Our findings indicate that feed supplementation with a multi-strain probiotic and clinoptilolite strengthens the intestinal barrier and stimulates cell regeneration. This is also supported by the reduction in the amount of excreted *Eimeria* oocysts observed in the experiment (Figure 3), as well as by the mRNA expression of genes responsible for the functioning of TjJ. The use of a multi-strain probiotic in the diet of chickens also leads to modification of the composition of the intestinal microbiome, which helps to inhibit the multiplication of conditionally pathogenic bacteria and stimulates the local immune system, improving the repair and regeneration mechanisms of the intestinal epithelium [23–53]. The reduction in intestinal permeability is not only due to the mitigation of damage to the mucosa caused by the multiplication of protozoa in the enterocytes, but is also the effect of the probiotic and clinoptilolite on the synthesis of proteins making up tight junctions, which regulate paracellular permeability. Clinoptilolite supplementation reduces zonulin synthesis and hence improves intestinal barrier integrity, perhaps by interacting with intestinal bacteria [54–56]. The transmembrane protein occludin performs an important function in the intestinal barrier, stabilising tight junctions (TJ) and thereby ensuring their structural integrity [57]. When microorganisms selectively disturb tight junction complexes formed by the plasma membrane proteins occludin and zonula occludens (ZO), the transepithelial electrical resistance (TER) of the epithelial cell layer rapidly decreases, resulting in increased paracellular permeability [58]. This hypothesis may be supported by Teng et al. [11], who showed that *E. maxima* infection decreases the mRNA expression of OCLDN, ZO_1, and CLDN2. Similar observations were made by Utech et al. [59] in a model of intestinal inflammation caused by *Eimeria* spp. infection, which was accompanied by an increase in TNF-α and IFN-γ concentrations and a decrease in the expression of OCLDN and ZO-1. These results were not confirmed in our experiment, in which birds infected with *E. tenella* (group VI) showed higher ($p \leq 0.05$) mRNA expression of OCLDN. The results of research conducted in a human model indicate that TNF-α and IFN-γ reduce gene expression of tight junction proteins, but the composition of the tight junction is highly regulated, and the loss or redistribution of one member of the intercellular junction

may be compensated for through the upregulation of another tight junction protein [60]. Therefore, the increase in OCLDN mRNA expression observed in group VI may be due to the activation of the intracellular transcription pathway of genes for tight junction proteins dependent on TNF-α and IFN-γ. These results, in combination with the clinical course of coccidial infection, enteritis observed in necropsied birds, and high lesion scores (LS) (Tables 4 and 7), suggest the promotion of the Th1 cellular immune response phenotype during *E. tenella* infection. Synthesis of cytokines, e.g., TNF-α, resulting from local intestinal inflammation induced by *Eimeria*, can be assumed to stimulate the body's defence mechanisms and promote regeneration of the intestinal epithelium, leading to increased expression of OCLDN. Similar results were obtained by Wickramasuriya et al. [61], who reported that chickens infected with *E. acervulina* that received a probiotic containing *B. subtilis* in their feed showed increased expression of OCLDN, which helps to stabilise the intestinal barrier. In our study, however, chickens infected with *E. tenella* that received EM Bokashi® and clinoptilolite with their feed showed reduced expression of OCLDN in the caecum. Therefore, it can be assumed that both components of the feed additive take part in regulating the anti-parasitic response, stimulating mechanisms responsible for the regeneration of caecal epithelial cells, and leading to the restoration of the function of the intestinal barrier and the preservation of homeostasis within the intestinal wall.

Paracellular, they take part in cell polarization, cellular adhesion, and migration of cells, including leukocytes [62–65] ($p \leq 0.05$). Our results showed that the expression of JAM-2 mRNA was also significantly increased ($p \leq 0.05$) during *E. tenella* infection in the groups of infected birds receiving a probiotic and clinoptilolite in their feed. However, the level of its expression in these groups was lower by half compared to infected birds that received a basal diet, which demonstrates the protective function of the probiotic and clinoptilolite for the intestinal epithelium. These results demonstrate not only the protective role of these formulations for the intestinal epithelium but also the preservation of intact tight junctions between the enterocytes, which prevent large molecules that can disturb homeostasis from passing through the intestinal barrier. The results for the CLDN-1 gene were similar. ($p \leq 0.05$). The CLDN family of proteins, such as JAM-2, is responsible for the formation of tight junctions between cells, the apposition of cell membranes, and paracellular transport [62]. The low expression of the CLDN family and JAM-2 genes noted in the groups of birds receiving the probiotic and clinoptilolite is indicative of the neutralising effects of these compounds on *Eimeria tenella* in the caecum. In contrast, the significant increase ($p \leq 0.05$) in the expression of this gene in the group of infected birds (group VI) suggests that repair processes are activated in the junctions between enterocytes, which were damaged by *E. tenella* sporozoites. Increased expression of CLDN-1 may also be associated with an excessive inflammatory response to infection by *E. tenella*, which leads to a sharp increase in the concentrations of pro-inflammatory cytokines TNF-α and IFN-γ [63,64]. Poritz [65] and Utech [59] showed that during the inflammatory process, these cytokines increase the expression of CLDN1 while decreasing the expression of OCLDN and ZO1. These results were partially confirmed in our study, in which the birds infected with *E. tenella* (group VI) showed an increase in the expression of CLDN-1 and JAM-2 and a decrease in that of ZO-1. The increase in expression of the CLDN2 gene in the group of infected birds (group VI) indicates a leaky intestinal epithelium and the presence of inflammation induced by *E. tenella* infection. Similar observations were made by Pham and Hatabu [6], who showed high mRNA expression of CLDN-2 in chickens infected with *E. tenella*, indicating inflammation in the intestine and disturbances of transport by enterocytes.

Another protein, zonulin, is a physiological modulator of the function of tight junctions and is responsible for the transepithelial transport of ions and fluids between the intestinal lumen and the bloodstream, thereby regulating the permeability of the intestines [66–69]. Teng et al. [8] reported reduced mRNA expression of ZO-1 and ZO-2 in chickens infected with *E. maxima*. Similar results were obtained in our study. The decrease in mRNA expression of ZO-1 and ZO-2 in chickens infected with *E. tenella* (group VI) suggests a

physiological loss of junctions between enterocytes. The low expression of genes noted in the present study may also be linked to the bloody diarrhoea observed in chickens infected with *E. tenella*, confirming the occurrence of intestinal inflammation, which was also reported by Pham and Hatabu [6].

The low mRNA expression of MUC-2 encoding the major mucin produced by goblet cells, shown in the caecal mucosa of the chickens infected with *E. tenella* (group VI), is associated with increased intestinal inflammation and impaired regeneration of the mucus layer [70–72]. The correlation of mRNA expression of MUC-2, ZO-1, ZO-2, CLDN-1, and CLDN-2 in this group of birds indicates a total loss of intercellular junctions and impeded transport between cells as well as between cells and the intestinal lumen, which is conducive to bacterial infections exacerbating intestinal inflammation induced by *E. tenella*. The chickens receiving a feed additive consisting of EM Bokashi®, together with clinoptilolite, showed a gradual increase in the expression of the MUC-2 gene, which reached its highest level at 42 days of age. It should be noted that probiotic bacteria, e.g., *Lactobacillus* or *Bacillus* spp., can bind to specific receptor sites on enterocytes and stimulate MUC2 synthesis [70,73,74]. It can be hypothesised that the EM Bokashi® and clinoptilolite formulation used in the diet of poultry contributed to increased resistance to enteric pathogens, including *Eimeria* spp., and improved feed conversion, in part by upregulating MUC2 expression, which increased mucin production and protected the intestine against morphological changes following infection with *E. tenella*.

One of the effects of damage to enterocytes by coccidia is the release of reactive oxygen species (ROS), which impair the function of the intestinal barrier [75–77]. Preservation of the balance between the production and removal of free oxygen radicals depends in part on enzymes with antioxidant properties, such as superoxide dismutase (SOD), catalase (CAT), and haem oxygenase (HMOX1/HO-1) [78,79]. In the present study, the highest mRNA expression ($p \leq 0.05$) of SOD was noted in chickens infected with *E. tenella* (group VI), while in the birds receiving the preparation containing EM Bokashi® and clinoptilolite, its expression did not differ from that noted in control. Different results were obtained by Elmahallawy et al. [80]. The results of our study show that the use of a feed additive containing a probiotic and clinoptilolite in the diet of poultry with experimentally induced coccidiosis reduces ROS release by reducing the severity of the infection, intestinal inflammation, and the degree of tissue damage in the caecum, which improves the overall health of birds. It is worth noting that probiotic bacteria used in feed additives, e.g., lactic acid bacteria, owing to their ability to adhere to the intestinal mucosa, can supply exogenous antioxidant enzymes to the inflamed tissue lying below and, in this way, inactivate ROS by transforming superoxide anion radicals into the less toxic H_2O_2, thereby limiting inflammation [81,82]. The low SOD concentrations obtained in the present study may therefore indicate homeostasis of mechanisms responsible for combating oxidative stress in *E. tenella* infection. A similar relationship has been shown for the degree of expression of the SOD1 and CAT genes in the duodenal mucosa of chickens infected with *E. acervulina* receiving a diet with *B. subtilis*-cNK-2 [61].

The reverse relationship was shown for the mRNA expression of CAT, which was highest in poultry infected with *E. tenella* and receiving a diet with a probiotic together with clinoptilolite. The results are supported by research by Wickramasuriya et al. [61], who showed no statistically significant differences in CAT expression in the intestines of poultry, while in poultry infected with *E. acervulina* and fed a diet supplemented with *B. subtilis*-cNK-2, expression of CAT and HO-1 increased in the spleen. Interesting results were obtained in the analysis of the mRNA expression of haem oxygenase HO-1. Increased expression of HO-1 is usually noted in conditions of cell exposure to oxidative stress [83]. The protective activity of this enzyme involves limiting the damage caused by oxidative stress, ischaemia, or inflammation, while the mechanism of protective activity is varied and multifaceted [77]. These results are consistent with those published by Wickramasuriya et al. [61], who also showed increased expression of the HMOX1 gene in poultry infected with *E. acervulina*. These findings confirm the cytoprotective effect of haem oxygenases on intestinal cells,

expressed as a reduction in oxidative stress and the severity of intestinal inflammation as well as the maintenance of enterocyte integrity, which is also evidenced by the clinical course of infection in the birds. Our results showed that the formulation consisting of EM Bokashi® and clinoptilolite in the diet of poultry enhanced antioxidant processes in the caecal mucosa and reduced oxidative stress induced by *E. tenella* infection, reducing the risk of damage to the enterocytes.

Eimeria spp., through invasive activity and destruction of the intestinal epithelium, leads to malabsorption in poultry and clinical gastrointestinal symptoms, including bloody diarrhoea. In infected birds, there is a secondary reduction in feed intake and conversion and a decrease in body weight gains, which ultimately leads to developmental disorders and death [64,84–86]. The chickens infected with *Eimeria tenella* had a low growth rate, expressed as a reduction in BWG and FI in comparison to the uninfected control and the other experimental groups. Our study showed that the use of EM Bokashi® and clinoptilolite in the diet significantly ($p \leq 0.05$) affected the growth rate of chicken broilers infected with *E. tenella*, expressed as an increase in BWG and FI. However, previously published data on the effect of probiotics in the diet of poultry on the health and production parameters of birds infected with *Eimeria* spp. are conflicting [87–90]. Similarly, divergent results have been published for zeolite [28,29,91]. Our results are largely consistent with those published by Giannenas et al. [92,93], Zhou et al. [91], and Timmerman et al. [94] and indicate that body weight gains in broilers receiving a diet with probiotics and zeolite and infected with *E. tenella* are significantly higher than in birds fed a standard diet. In addition, the use of the composite probiotic formulation in our experiment in the first period after hatching favours competitive colonisation of the intestinal epithelium with probiotic microbes, even in birds infected with *E. tenella* at 14 days of age. The microbes contained in the probiotic prevent the adhesion of pathogenic agents to the intestinal epithelium and modulate the expression of genes in epithelial cells, taking part in the immune response against the development of infections that negatively affect production and health parameters [95]. The use of the multi-strain and multi-species probiotic formulation allows the effective microorganisms contained in it to act in different parts of the intestine and present varied mechanisms of action. This makes it possible to eliminate the negative effects of an *Eimeria* infection. In addition, the clinoptilolite used in the experiment, together with the probiotic preparation, is known to be rich in macro- and microelements essential for the growth and development of the body. In ionised form, these elements have been shown to be quickly absorbed and distributed to various organs, beneficially affecting the metabolic processes taking place in them and their biological functions [96,97], which translates to improved production parameters.

The use of the feed supplement composed of a probiotic and clinoptilolite in the diet of poultry caused a significant ($p \leq 0.05$) reduction in the number of *E. tenella* oocysts in the faeces and caecum in comparison to the group of infected birds, the other experimental groups, and the control. Similar observations were reported by Giannenas et al. [93], who showed that the body weight of chickens infected with *E. tenella* receiving feed with a probiotic was higher than or similar to that of infected birds that received a coccidiostat, while the number of oocysts in the faeces was much lower than in the control group of infected birds fed a standard diet. Similar results were obtained by Lee et al. [98] in a study of poultry fed a diet with a probiotic supplement containing *Pediococcus acidilactici*. The beneficial effect of the probiotic as a feed additive was expressed in part as increased resistance to experimental infection with *E. acervulina*, increased body weight gains in comparison with infected birds fed the basal diet, and a decrease in excretion of oocysts. Similar relationships were demonstrated in our study. The mechanism of coccidiostatic action of the compounds most likely relies on competition between probiotic organisms and oocysts for access to specific sites of colonisation of the intestinal epithelium. In our opinion, the inclusion of clinoptilolite in the feed supplement plays a role as well. These aluminosilicates are known to have a strong adsorption capacity and are able to adsorb bacteria, toxic substances, and other harmful compounds in the intestines, which promotes

the excretion of oocysts [99–101]. Another advantage of clinoptilolite added to feed is its uniform distribution over the surface of the intestinal mucosa, owing to which it forms a kind of protective layer that reduces colonisation of the intestine by oocysts and damage to the mucosa by *Eimeria* spp. Nevertheless, the exact mechanism of action of probiotics and clinoptilolite at the molecular level and their effect on the colonisation of the intestinal epithelium by oocysts and the growth of animals are not fully understood and require further research.

The analysis of lesion scores (LS) in the experiment showed that replication of the parasite and the pathological changes found in the intestines, especially in the caecum, were statistically significantly ($p \leq 0.05$) more severe in the chickens infected with *E. tenella* (group VI) in comparison with the other experimental groups and the control. This was confirmed by the clinical observations of the birds in the infected group, in which symptoms of bloody diarrhoea and inhibition of body weight gains and feed intake were noted. The development of pathological changes in the caecum following infection with *E. tenella* is complex and is the combined effect of the accumulation of parasites mechanically damaging the enterocytes and the inflammatory immune response. Conway et al. [102] and Soutter et al. [103] showed that 4000 oocysts of *E. tenella* are sufficient to induce visible anatomopathological changes in the intestines affecting the production and health parameters of birds, while higher levels also cause an increase in mortality [102,103]. We made similar observations in our study, in which the number of oocysts in both the faeces and the caecum exceeded 4000 in the control group infected with *E. tenella*.

The synergistic activity of the probiotic formulation in combination with clinoptilolite, affecting digestion, nutrient utilisation, and nutrient metabolism, limits colonisation of the intestinal mucosa by *E. tenella* and the pathogenic effects of oocysts [104], thereby improving the survival of birds and reducing the extent of damage caused by the parasite in the gastrointestinal tract [104]. The reduced severity of lesions in this group of birds can be linked to the antimicrobial properties of the feed additive, based on its competitive capacity to eliminate microorganisms from the intestinal lumen [105,106] and a reduction in the effects of conditionally pathogenic bacteria on the intestinal epithelium [107]. These processes effectively inhibit the adhesion of oocysts to the intestinal barrier, their penetration of the epithelium, and their pathogenic effects. The improvement in growth parameters and overall health observed in this study additionally indicates mitigation of the clinical course of *E. tenella* infection in birds fed a diet supplemented with a probiotic and clinoptilolite.

5. Conclusions

The combined use of effective microorganisms (EM) in a multi-strain probiotic (Bokashi®) and clinoptilolite helps to maintain the intestinal barrier in chickens. The use of these compounds in *E. tenella*-infected birds is indicative of their neutralising effects on the parasite in the caecum. This prevents damage to the intestinal epithelium and demonstrates the protective role of enterocytes. Moreover, they inhibit the development of inflammation and prevent pro-inflammatory molecules from penetrating the intestinal mucosa. An additional benefit is improved feed conversion and stimulation of growth in broilers, partly due to the increase in mucin production and protection of the intestine against morphological changes following infection with *E. tenella*. The formulation used in this study also enhanced antioxidant processes in the caecal mucosa and reduced oxidative stress induced by *E. tenella* infection. The use of effective microorganisms (EM) in a multi-strain probiotic (Bokashi®) and clinoptilolite in the diet of poultry with experimentally induced coccidiosis limits ROS release by reducing the severity of the infection, intestinal inflammation, and the degree of tissue damage in the caecum, thereby improving the overall health of birds.

Author Contributions: Conceptualization, A.C., Ł.S.J. and M.H.; methodology, Ł.S.J., M.K., A.M., M.H. and S.N.; software, S.G. and A.R.; validation, A.R.; formal analysis, Ł.S.J. and Z.G.; investigation, A.C., Ł.S.J. and M.K.; resources, S.G. and A.R.; data curation, Ł.S.J.; writing—original draft preparation, A.C. and Ł.S.J.; writing—review and editing, Ł.S.J. and A.M.; visualization, A.R.; supervision,

Z.G.; project administration, Ł.S.J. and Z.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the University of Life Sciences in Lublin, Poland, project No. WKE/MN-1/WET/20.

Institutional Review Board Statement: All procedures involving animals were in compliance with the ethical standards of the institution at which the experiment was conducted. All procedures used during the research were approved by the Local Ethics Committee for Animal Testing at the University of Life Sciences in Lublin, Poland (approval no. 11/2021 of 1 March 2021).

Data Availability Statement: All data generated or analyzed during this study are included in this published article and are available on request from the corresponding author.

Conflicts of Interest: The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria, educational grants, participation in speakers' bureaus, membership, employment, consultancies, stock ownership, or other equity interest, expert testimony, or patent-licensing arrangements) or non-financial interest (such as personal or professional relationships, affiliations, knowledge, or beliefs) in the subject matter or materials discussed in this manuscript.

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