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Regulation of Inorganic Zinc Supplementation on Intestinal Absorption, Metabolism, and Muscle Development in Broilers Fed Low-Protein Diets

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Abstract: The issue of nitrogen fertilizer contamination resulting from high-protein diets can be effectively solved by adopting low-protein diets. The objective of this study was to investigate the effects of inorganic zinc supplementation in low-protein diets on 42-day-old broilers across a multitude of parameters. To determine the optimal dose of inorganic zinc in broiler diets with a 1.5% reduction in crude protein, 1-day-old Arbor Acres broilers (n = 270) were randomly assigned to five groups, each containing 54 broilers. Our results revealed that inorganic zinc supplementation at levels of 130 mg/kg elevated growth performance and carcass traits ($p < 0.05$). It also significantly increased the ratio of intestinal villi heights to crypt depths ($p < 0.001$), changed intestinal morphology, and significantly increased albumin content in serum ($p < 0.05$). Furthermore, analysis of mRNA expression showed that 130 mg/kg and 150 mg/kg of inorganic zinc improved the myogenic differentiation involved in muscle development, as well as intestinal tight junction and liver metallothionein capacity ($p < 0.001$). Additionally, these groups exhibited lower zinc excretion compared with other treatments ($p < 0.001$). In summary, our findings suggest that inorganic zinc supplementation in low-protein diets holds the potential to support muscle and intestinal development in broilers, presenting a viable nutritional strategy.

Keywords: broilers; inorganic zinc; low-protein diets; muscle development; zinc absorption

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1. Introduction

Given the capacity of common protein diets to satisfy the nutritional requisites of broilers and promote their growth, producers exhibit a preference for utilizing high-protein diets to sustain optimal performance in poultry production. Excessive protein intake during animal growth can result in calcium loss, kidney damage, and increased risk of disease [1]. Additionally, the accelerating expansion of the livestock and poultry breeding industries has inadvertently contributed to environmental degradation, primarily through the substantial emission of nitrogenous compounds [2]. Low-protein diet technology stands as an effective strategy to overcome the challenges mentioned above. Previous research confirmed that a 3% decrease in the crude protein content of broiler feed did not adversely affect their growth performance [3]. However, indiscriminately reducing the protein content in poultry diets can negatively affect the growth of broilers [4]. Yet, decreasing the crude protein content and concurrently supplementing with adequate amounts of synthetic amino acids has been demonstrated to improve protein utilization and enhance the production performance of livestock and poultry [5]. Therefore, it is worth considering whether the addition of

trace elements to low-protein diets can rectify problems associated with stunted growth in livestock and poultry. This approach demands careful and systematic application of low-protein diets, ensuring a precise adjustment of the protein content in the feed to fulfill nutritional requirements and meet environmental standards. Concurrently, there exists a discernible gap in our understanding of the nutritional standards of the trace element zinc in protein diets, particularly in relation to its influence on growth and metabolic processes in broiler chickens, which needs further exploration [6].

Zinc plays an indispensable role in the physiology of broilers, manifesting in three predominant functions: catalytic, regulatory, and structural [7]. It extends its influence over a plethora of enzymatic reactions, crucially enhancing avian appetite, optimizing feed intake, and, subsequently, augmenting overall feed conversion efficiency [8,9]. Furthermore, zinc is implicated in the intricate regulation of protein metabolism and interactions with various amino acids [10]. Within the domain of the small intestines, zinc is capable of forming stable complexes with amino acids. These complexes exhibit resilience against the inhibitory effects of intestinal phytic acid [11], thereby significantly promoting the bioavailability and utilization of zinc [12]. This complex interaction highlights how crucial zinc is in improving the health and growth of broilers. It emphasizes the need for a detailed and clear understanding of zinc's roles in the body and its nutritional benefits to ensure the best outcomes in poultry farming.

Zinc plays a pivotal role in bolstering immunity, subsequently fostering growth in broilers [13]. The concentration of zinc in animal blood and tissues serves as a tangible indicator of its absorption and utilization within the body. There is a positive correlation between the levels of zinc in animal diets and serum zinc levels, which is indicative of robust growth and development in the animals [14]. Intestinal damage has been linked to a decrement in production performance [15], and zinc has been shown to facilitate the proliferation of intestinal crypt cells [16], aiding in the repair of epithelial cells and sustaining the integrity of the intestinal barrier's structure and function. Alterations in intestinal permeability may be influenced by the regulation and functionality of tight junction proteins such as Claudin-1, Occludin, and ZO-1 [17]. The inclusion of zinc supplements in diets has been found to upregulate the expression of these tight junction proteins, thereby preserving the integrity of the intestinal epithelial barrier [18]. The metallothionein (MT) protein family is crucial to the metabolism of zinc. MT binds with zinc within cells through sulfhydryl bonds, playing a critical role in maintaining cellular zinc homeostasis. It also serves as a valuable marker for assessing the body's zinc storage capacity. Beyond its role in zinc metabolism, MT is involved in modulating various oxidative stress responses and immune functions within the body [19]. The myogenic regulatory factors family is instrumental in the development and regeneration of muscle cells, participating in every stage of muscle development. This includes key factors such as myogenic differentiation factor 1 (MyoD1), myogenic factor 5 (Myf5), myogenic factor 6 (Myf6), and Myogenin [20]. The regulation of expression by these myogenic regulatory factors is essential for the development of skeletal muscle, positioning them as primary genes for evaluating the production traits of livestock and poultry meat. This indicates the necessity of understanding the multifaceted roles of zinc in broiler nutrition and the development of optimal poultry production.

Previous studies have demonstrated that incorporating either $ZnSO_4$ or ZnO into the diet of broilers can effectively reduce total nitrogen loss in their manure and diminish microbial enzyme activities associated with ammonia production [21]. Given their distinct bioavailability, the synergistic utilization of these two inorganic zinc sources in our study holds promise for enhancing outcomes for broilers. Nevertheless, the optimal quantity of inorganic zinc in low-protein diets remains to be established. Referencing the Newly Proposed Total Maximum Contents (NPMC) standards, the inorganic zinc requirement for optimal broiler production performance is pinpointed to range from 70 to 140 mg/kg [22]. As broiler breeds continue to evolve and adapt to their environments, it becomes imperative to scrutinize the potential impacts of zinc (be it within or exceeding the recommended levels) on the growth and development of broilers. This nuanced approach ensures alignment

with contemporary agricultural practices aiming to optimize broiler production while maintaining environmental protection.

Therefore, we explored how gradually increasing amounts of inorganic zinc in a low-protein diet affect broiler production, serum biochemistry, meat quality, intestinal development, and zinc residue. Our study aims to provide a theoretical basis for establishing zinc nutrition standards in low-protein diets for broilers, contributing to reduced nitrogen and mineral excretion.

2. Materials and Methods

2.1. Ethical Approval

The experimental protocol used in this study was approved by the Animal Ethics and Welfare Committee of Jilin University and followed the requirements of Jilin University and the state for the ethical welfare of laboratory animals (approval number: SY202107300). The feeding conditions were strictly in accordance with GB14925, People's Republic of China.

2.2. Broilers, Feeding, and Management

In this study, we randomly selected 270 one-day-old Arbor Acres (AA) broilers from a local farm; each broiler had an initial average weight of 42.6 ± 0.59 g. These broilers were then allocated into five treatment groups, with 54 broilers per group. Over a 42-day experimental period, the broilers were provided with three distinct stages of basal feed: early (0–14 days), middle (15–28 days), and late (29–42 days), the compositions of which are described in Table 1. This trial implemented a low-protein diet in accordance with the guidelines set out in item NH2021202205, aiming to evaluate the effects of supplementing different concentrations of inorganic zinc (70, 90, 110, 130, or 150 mg/kg) in the low-protein-treated groups following the newly established NPMC standards [22]. The zinc premix utilized comprised zinc sulfate ($ZnSO_4 \cdot 7H_2O$, 23% zinc content) and feed-grade zinc oxide (ZnO, 80% zinc content) blended in a 2:1 ratio; both were of feed-grade quality. The actual zinc content in the feed is specified in Table 2. All broilers were raised flat on iron nets in the same environment with unrestricted access to water and feed. Throughout the experiment, broilers were exposed to light for 23 hours per day. Daily management tasks ensuring routine disinfection and immunization of the coop were conducted to maintain consistent rearing conditions for all broilers. Strict hygiene and optimal environmental health conditions were upheld to support the broilers' growth and were maintained throughout the trial. A blower was used to maintain a regulated temperature in the laboratory room for the duration of the experiment.

Table 1. Ingredients and nutrient composition of diets at each feeding stage of AA broilers.

Feed Ingredients (%)	Feeding Stage		
	0–14 Days	15–28 Days	29–42 Days
Corn	61.21	69.39	73.08
Soyabean meal	31.70	23.50	20.10
Soya oil	0.80	1.10	1.50
Corn protein flour	2.50	2.50	2.40
Dicalcium phosphate	1.59	1.66	1.34
Stone powder	0.98	0.54	0.41
NaCl	0.28	0.30	0.21
DL-Methionine	0.21	0.19	0.18
Baking soda	0.21	0.21	0.20
L-Lysine hydrochloride	0.10	0.17	0.18
Trace element premix [1]	0.10	0.10	0.10
Choline chloride-50	0.10	0.10	0.10
Vitamin–mineral premix [2]	0.05	0.05	0.05
12% salinomycin	0.05	0.05	0.00
Thermostable phytase	0.01	0.01	0.01
L-Threonine	0.00	0.02	0.03

Table 1. *Cont.*

Feed Ingredients (%)	Feeding Stage		
	0–14 Days	15–28 Days	29–42 Days
Compound enzyme preparation	0.01	0.01	0.01
Substitute antibodies [a]	0.10	0.10	0.10
Total %	100	100	100
Nutrient composition Chemical analysis [3] (%)			
Crude protein (CP) (DM%)	21.31	17.96	16.47
Coarse ash (DM%)	5.73	5.32	5.20
Crude fat (DM%)	2.16	2.47	2.22
Crude fiber (DM%)	3.81	3.54	3.77
Moisture (DM%)	14.59	15.10	13.05
Nitrogen-free extract (DM%)	63.36	67.51	69.87
Calcium (DM%)	1.04	1.31	1.67
Phosphorus (DM%)	1.34	1.38	1.59
Lysine (DM%)	0.70	0.80	0.90
Methionine (DM%)	1.57	1.76	1.91
Zn (mg/kg) [b]	110	110	110
ME (MJ/kg)	11.67	12.01	12.31

[1] Supplied per kg of trace element premix: Cu (copper sulfate), 15 mg; Fe (ferrous sulfate), 52 mg; Mn (manganese sulfate), 115.2 mg; I (potassium iodide), 1.14 mg; Se (sodium selenite), 0.30 mg. [2] Supplied per kg of mineral premix: trans-retinol (A), 50,000K IU; cholecalciferol (D3), 12,500K IU; α-tocopherol acetate (E), 90K IU; VK3, 15,000 mg; hiamine (B1), 10,000 mg; riboflavin (B2), 35,000 mg; pyridoxine (B6), 15,000 mg; vitamin B12, 100 mg; nicotinic acid, 150,000 mg; Calcium pantothenate, 50,000 mg; folic acid, 7000 mg; biotin, 350 mg. [3] Nutrient level indicators are calculated values. [a] Substitute antibodies are commercial products composed of essential oils and acids. The additive content includes raw materials such as citric acid, cinnamaldehyde, thymol, carvacrol, eugenol, calcium lactate, and ethoxyquin, with Maifan stone serving as the carrier. [b] The Zn value presented in the table indicates the baseline level of total zinc for each feeding stage, with the specific zinc content for each group provided in Table 2.

Table 2. Inorganic zinc content of feed at different feeding stages.

Stage	Content of Inorganic Zinc in a Low-Protein Diet					SEM
	70 mg/kg	90 mg/kg	110 mg/kg	130 mg/kg	150 mg/kg	
Day 0–14	72.82	88.17	115.50	127.13	143.61	1.979
Day 15–28	69.55	83.21	115.20	128.35	143.16	0.768
Day 29–42	72.37	84.21	106.23	126.61	145.99	2.389

2.3. Growth Performance and Carcass Trait Analysis

Body weights and weekly feed consumption of the broilers were systematically recorded at the same time (at noon) on days 0, 7, 14, 21, 28, 35, and 42 of the experiment. This data collection facilitated the computation of key performance metrics such as the average daily gain (ADG), average daily feed intake (ADFI), and the feed-to-gain ratio (F/G). Upon reaching 42 days of age, a total of 6 broilers were selected from each group (for a total of 30 broilers) to be slaughtered. In preparation for this, the selected broilers underwent a 24 h fasting regimen coupled with water deprivation. Their live weights were measured 12 h preceding euthanasia. Concomitant with the euthanasia procedure, we procured and subsequently weighed tissue samples from distinct anatomical regions. The carcass rate, semi-chamber rate, total chamber rate, pectoral muscle rate, leg muscle rate, and abdominal fat rate of the pectoral muscles, leg muscles, liver, and jejunum were determined based on the terminology and measurement statistical method for poultry production performance in China (NY/T 823-2004). Following collection, all samples were promptly subjected to flash-freezing in liquid nitrogen and then preserved at a temperature of −80 °C. This meticulous sample preservation strategy ensures the integrity and reliability of our biological specimens for future analytical endeavors.

2.4. Meat Quality Determination

Meat color was quantitatively evaluated using a chromameter (OPTO-STAR, MATTHAUS, Poettmes, Germany), yielding values in accordance with the International Commission on Illumination (CIE) LAB system, particularly for lightness (L*). The meat color spectrum captured ranged upwards from light, categorized by L* values exceeding 53. Muscle pH was measured after 45 min of slaughter using a carcass meat pH direct tester (pH-STAR, MATTHAUS, Poettmes, Germany) that was meticulously calibrated at pH values of 4.0 and 7.0 at ambient room temperature. The water-holding capacity (WHC) of the meat was determined following a previously described method [23]. A muscle sample, approximately 3.0 g in weight, was placed in a water-holding capacity tester (RH-1000, Guangzhou Runhu Instruments Co., Ltd., Guangzhou, China) and subjected to a pressure of 2000 psi for one minute, sandwiched between 16 pieces of 125 mm filter papers. Water release from meat fibers was assessed using a hydraulic analyzer. The WHC was then calculated as the ratio of the weight lost to the initial weight of the sample. The pectoralis muscle and leg muscle samples, with an average sample weight of 8.8 g, were subjected to thermal treatment, sealed in plastic bags, and immersed in a 96 °C water bath for 30 min. After cooling to room temperature, the samples were sliced thrice in parallel to the myofibers, and the shear force was measured in triplicate using a digital muscle tenderness meter (C-LM4, Tenovo, Beijing, China). To evaluate drip loss, pectoralis and leg muscle samples with an average weight of 12.3 g were sealed in plastic bags, ensuring no contact between the sample and the bag. The samples were then suspended with a wire hook and refrigerated at 2–4 °C. After 24 h, the samples were wiped dry and weighed once again, with drip loss calculated and expressed as a percentage of the initial weight following the methodology outlined by Hu et al. [24].

2.5. Preparation and Analysis of Blood Samples

Blood samples were collected from the jugular veins of 30 broilers (6 broilers per group) through phlebotomy during slaughter and placed directly into serum centrifuge tubes. The samples were then centrifuged at 3500 rpm for 10 min to facilitate separation of the sera, which were then transferred into 2 mL centrifuge tubes. These sera samples were preserved at −20 °C until they were required for enzyme analysis. The levels of alkaline phosphatase (AKP), total protein (TP), albumin (ALB), and globulin (GLO) present in the sera were quantified using an automatic biochemical analyzer (MS-880B, Medicalsystem Biotechnology Co., Ltd., Ningbo, China).

2.6. Tissue Sampling for Intestinal Morphological Observation

Jejunal tissue samples were meticulously harvested at the time of slaughter, gently rinsed with 0.9% saline solution, and subsequently fixed in a 4% paraformaldehyde solution in preparation for intestinal morphology assessment using hematoxylin and eosin (H&E) staining. Following this, the samples underwent meticulous paraffin wax embedding, precise sectioning, and then staining with H&E. For an in-depth analysis, three representative fields from the intestinal sections were selected and examined under an Olympus light microscope at 40× magnification. During this examination, measurements of villus height (VH), crypt depth (CD), and the ratio of villus height to crypt depth (V/C) were conducted for each field of view using advanced slide image analysis software. Prior to the microscopic examination, the jejunal tissue sections were carefully dewaxed, rehydrated, and stained using hematoxylin and eosin. This was then followed by a dehydration process. The Olympus light microscope was once again employed to scrutinize three representative areas of the intestinal sections at 40× magnification, capturing detailed images for analysis. Measurements of VH, CD, and the V/C for each field of view were taken, ensuring precision and accuracy through the use of sophisticated slide image analysis software.

2.7. Fecal Trace Elements

The acquired feed and manure samples were preserved at −20 °C prior to the quantification of their zinc content, which was meticulously carried out using an atomic absorption spectrophotometer (WFX-320, Beijing Beifen-Ruili Analytical Instrument Group Co., Ltd., Beijing, China). The samples were subjected to the analytical process, adhering to established protocols, and the spectrophotometer was rigorously calibrated to ensure precision in the ensuing analysis. To guarantee the reliability of our results, triplicate measurements were taken, and the results were subsequently expressed with reference to a standard curve derived from known zinc standards. The methodology adopted for both sample preparation and analysis was in strict accordance with procedures documented in the existing literature [25].

2.8. Total RNA Extraction, cDNA Synthesis, and Quantitative PCR

Total RNA was extracted from liver, muscle, and intestinal tissues using TRIzol™ Reagent (Invitrogen, MA, USA) following the manufacturer's protocol. The extracted RNA's quantity, purity, and integrity were evaluated using gel electrophoresis and a Nanodrop spectrophotometer (Nanodrop 2000, Thermo Fisher Scientific Inc., Shanghai, China). Reverse transcription was subsequently conducted using the FastKing RT Kit (with gDNase) and Fasting cDNA Strand 1 Synthesis Kit (Tiangen Biotech Co., Ltd., Beijing, China). PCR primers were designed on the NCBI website and synthesized by Jilin Kumei Biotechnology. The sequences of the primers are listed in Table 3.

Beta-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as housekeeping genes for normalizing the results. The qPCR reactions were performed using TransStart® Top Green qPCR SuperMix (TransGen, Beijing, China) on an Agilent Mx3005P instrument (Agilent Technologies, Beijing, China). The PCR reaction mixture consisted of 0.4 μL of cDNA forward primer (10 μM), 0.4 μL of reverse primer (10 μM), and 10 μL of 2× TransStart® Top Green qPCR SuperMix, with the cDNA and nuclease-free water volumes adjusted based on cDNA concentrations to reach a total volume of 20 μL. The thermal cycling conditions included an initial denaturation at 94 °C for 30 cycles followed by 94 °C for 5 cycles. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. The mRNA expression levels of hepatic metallothionein (MT-1), myogenic factor 5 (Myf5), myogenic factor 6 (Myf6), myogenic differentiation 1 (MyOD), myogenin (MyOG), claudin-1, and zonula occludens-1 (ZO-1) were assessed following the outlined procedure. All genes mentioned in the article demonstrated amplification efficiencies above 95%. Each sample underwent triplicate analyses and the entire experiment was replicated four times for each sample.

Table 3. Sequences and parameters of PCR primers.

Gene Names	Accession No.	Primer Sequences (5′—3′)	Annealing Temp (°C)
Beta-actin	NM_205518	ATCCGGACCCTCCATTGTC AGCCATGCCAATCTCGTCTT	55
GADPH	NM_204305.1	GGAGAAACCAGCCAAGTAT CCATTGAAGTCACAGGAGA	55
Myf5	NM_001030363.2	TTCGAGACCTTGAAGAGGTGC TGTACCTGATGGCGTTCCTC	55
Myf6	NM_001030746.3	GCTCTGAAAAGGCGGACTGT TCCTGCAGCCTCTCGATGTA	60
MyoD	NM_204214.3	ACCCAAAGCATGGGAAGAGT AGGCAGTATGGGACATGTGG	55
MyOG	NM_204305.1	GTGACCCTGTGCCCTGAAAG TCGATGGACACGGTTTTGCG	60
MT	NM_205275.1	CTCCTGCTCCTGTGCTGGGTCGTGC CGGTTCCTTGCAGACACAGCCCTT	55

Table 3. *Cont.*

Gene Names	Accession No.	Primer Sequences (5′—3′)	Annealing Temp (°C)
Claudin-1	NM_001013611.2	ATCCAGTGCAAGGTGTACGA ACCAACCAGACCCAGGAGTA	55
Occludin-1	NM_205128.1	TCTGTGCTGAGATGGACAGC TCCTCTGCCACATCCTGGTA	55
ZO-1	XM_413.773	CTTCAGGTGTTTCTCTTCCTCCTC CTGTGGTTTCATGGCTGGATC	55

Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Myf5, myogenic factor 5; Myf6, myogenic factor 6; MyOD, myogenic differentiation 1; MyOG, Myogenin; MT, Metallothionein; ZO-1, zonula occludens-1.

2.9. Statistical Analyses

All original data were analyzed using a completely randomized design, where each group served as a separate treatment group, and the broilers in each group were treated as independent individuals. The data collected from the experimental groups were analyzed using SPSS statistical software (version 27.0; IBM Corp., Armonk, NY, USA). To detect significant differences between the treatments, we used the Tukey test within the framework of one-way ANOVA. A *p*-value threshold of <0.05 was set to establish statistical significance.

3. Results

3.1. Inorganic Zinc Supplementation Content of Low-Protein Diets

The concentrations of Zn in the experimental groups, as determined through analysis, were found to be in close agreement with the Zn concentrations initially calculated following the incorporation of an additional inorganic zinc source into the diet (Table 2).

3.2. Effects of Inorganic Zinc Supplementation in Low-Protein Diets on Growth Performance of Broilers

As presented in Table 4, a comprehensive analysis of the growth performance of broilers at each age from 0 to 42 days showed that the growth performance of broilers was significantly enhanced at 0–42 days of age. Broilers fed low-protein diets fortified with inorganic zinc at levels of either 130 mg/kg or 150 mg/kg demonstrated increased BW at week 5 ($p < 0.001$), as well as an optimized F/G ($p < 0.05$). Notably, no significant disparities were detected between broilers in the 130 mg/kg and 150 mg/kg treatment groups. These observed trends in growth performance advocate for the inclusion of inorganic zinc at concentrations of 130 mg/kg or 150 mg/kg in the diets of broilers aged up to 42 days to foster enhanced growth.

Table 4. Effects of gradual addition of inorganic zinc to low-protein diets on broiler growth performance.

Items	Content of Inorganic Zinc in a Low-Protein Diet					SEM	*p*-Value
	70 mg/kg (*n* = 54)	90 mg/kg (*n* = 54)	110 mg/kg (*n* = 54)	130 mg/kg (*n* = 54)	150 mg/kg (*n* = 54)		
	Body weight gain (g)						
Week 1	143.67	145.08	146.10	146.64	152.65	0.780	0.103
Week 2	262.72	261.43	270.97	276.22	286.80	1.222	0.047
Week 3	334.26 ^{b}	338.55 ^{b}	337.26 ^{b}	373.26 ^{ab}	384.70 ^{a}	5.058	0.009
Week 4	493.70	491.67	504.92	506.25	568.49	7.571	0.149
Week 5	541.69^{c}	578.12 ^{bc}	590.25 ^{bc}	616.17 ^{ab}	676.89 ^{a}	10.260	<0.001
Week 6	683.00	648.60	671.77	639.39	690.78	9.102	0.812

Table 4. *Cont.*

Items	Content of Inorganic Zinc in a Low-Protein Diet					SEM	*p*-Value
	70 mg/kg (*n* = 54)	90 mg/kg (*n* = 54)	110 mg/kg (*n* = 54)	130 mg/kg (*n* = 54)	150 mg/kg (*n* = 54)		
				Feed intake (g)			
Week 1	170.43	170.92	175.33	167.83	176.27	1.786	0.347
Week 2	366.76	371.52	378.08	379.18	380.46	1.043	0.103
Week 3	556.76	549.55	534.46	575.40	598.06	4.389	0.167
Week 4	754.24	742.05	741.76	717.96	810.66	11.037	0.612
Week 5	1000.94	1038.47	1045.52	1011.64	1105.98	3.489	0.351
Week 6	1295.34	1202.36	1207.14	1083.97	1174.06	24.751	0.361
				FCR			
Week 1	1.19	1.18	1.20	1.15	1.15	0.012	0.290
Week 2	1.40	1.42	1.40	1.37	1.33	0.015	0.452
Week 3	1.66	1.62	1.59	1.54	1.55	0.015	0.239
Week 4	1.53 ^{a}	1.51 ^{ab}	1.47 ^{ab}	1.42 ^{b}	1.43 ^{b}	0.004	0.015
Week 5	1.85	1.80	1.77	1.64	1.63	0.034	0.039
Week 6	1.90 ^{a}	1.85 ^{ab}	1.79 ^{ab}	1.70 ^{b}	1.70 ^{b}	0.004	0.006

The data are expressed as means. Superscripts (a–c) with no common letters within a row show values that differ significantly ($p < 0.05$). Abbreviations: FCR, feed conversion ratio; SEM, Standard error of the mean.

3.3. Effect of Inorganic Zinc Supplementation in Low-Protein Diets on Carcass Traits and Meat Quality of Broilers

As shown by the slaughter performance metrics of 42-day-old broilers detailed in Table 5, no statistically significant variations were observed in dressed percentages, semi-eviscerated rates, and leg muscle rates across the different groups of broilers ($p > 0.05$). However, the groups supplemented with inorganic zinc at concentrations of 130 mg/kg and 150 mg/kg exhibited a noticeable upward trend in these parameters compared with the other groups. Notably, the eviscerated rate and breast muscle rate in the 150 mg/kg group were significantly elevated compared with those in the other groups ($p < 0.05$), while the abdominal fat rate was markedly reduced ($p < 0.05$). Interestingly, these trends did not present significant differences when juxtaposed with the 130 mg/kg group. These findings indicate the positive influence of supplementing a low-protein diet with 130 mg/kg or 150 mg/kg of inorganic zinc on the slaughter performance of broilers, highlighting its potential benefits for enhancing poultry production outcomes.

Table 5. Effects of gradual addition of inorganic zinc to low-protein diets on carcass characteristics of 42-day-old broilers.

Items	Content of Inorganic Zinc in a Low-Protein Diet					SEM	*p*-Value
	70 mg/kg (*n* = 6)	90 mg/kg (*n* = 6)	110 mg/kg (*n* = 6)	130 mg/kg (*n* = 6)	150 mg/kg (*n* = 6)		
Dressed percentage	90.3	90.2	90.4	92.1	91.5	0.57	0.058
Percentage of half-eviscerated yield with giblet	81.4	81.3	81.5	82.8	81.5	0.62	0.080
Percentage of eviscerated yield	69.2 ^{b}	69.9 ^{ab}	70.1 ^{ab}	72.3 ^{a}	70.8 ^{ab}	0.92	0.044
Percentage of breast muscle yield	22.1 ^{b}	23.5 ^{ab}	23.6 ^{ab}	24.7 ^{a}	24.0 ^{ab}	0.76	0.037
Percentage of leg muscle yield	22.9	23.9	24.0	24.8	24.3	0.98	0.197
Percentage of abdominal fat yield	2.8 ^{a}	2.8 ^{a}	2.6 ^{a}	2.3 ^{ab}	2.0 ^{b}	0.20	0.002

The data are expressed as means. Superscripts (a,b) with no common letters within a row show values that differ significantly ($p < 0.05$). SEM: Standard error of the mean.

The quality parameters of broiler chickens at 42 days of age are presented in Table 6. Across all groups, the pH values for both pectoral and leg muscles exhibited no significant differences ($p > 0.05$). Nevertheless, a notable reduction in the L* values of the pectoral muscle was observed in the groups supplemented with 150 mg/kg and 130 mg/kg of

inorganic zinc compared with the other groups ($p < 0.05$). Furthermore, the L* value of the leg muscle in the 130 mg/kg group was significantly lower than those of the remaining groups ($p < 0.001$), albeit not differing substantially from the 150 mg/kg group. Regarding shear force and drip loss, no significant variance was detected between the 130 mg/kg and 150 mg/kg groups. However, these groups exhibited significantly lower shear force and drip loss values while demonstrating a notably higher water-holding capacity in comparison to the other three groups ($p < 0.001$). In summary, the inclusion of 130 mg/kg or 150 mg/kg of inorganic zinc in the diet of 42-day-old broilers appears to enhance the quality parameters of the meat, contributing positively to the overall quality of broiler meat.

Table 6. Effects of gradual addition of inorganic zinc to low-protein diets on the quality of meat of 42-day-old broilers.

Items	Place	Content of Inorganic Zinc in a Low-Protein Diet					SEM	*p*-Value
		70 mg/kg (*n* = 6)	90 mg/kg (*n* = 6)	110 mg/kg (*n* = 6)	130 mg/kg (*n* = 6)	150 mg/kg (*n* = 6)		
Flesh (L*)	Pectorals	77.8 ^{a}	76.8 ^{a}	75.2 ^{ab}	70.1 ^{b}	70.5 ^{b}	2.17	<0.001
	Leg muscles	71.6	71.8	70.6	67.4	67.5	1.84	0.030
PH_{45min}	Pectorals	5.81	5.92	5.96	6.09	6.06	0.170	0.391
	Leg muscles	6.21	6.21	6.26	6.29	6.39	0.080	0.309
Shearing force	Pectorals	30.3 ^{a}	28.4 ^{ab}	28.0 ^{ab}	24.6 ^{bc}	22.9 ^{c}	1.10	<0.001
	Leg muscles	27.6 ^{a}	26.1 ^{a}	25.5 ^{ab}	21.9 ^{b}	22.4 ^{b}	1.19	<0.001
Water holding capacity	Pectorals	17.5 ^{c}	19.2 ^{bc}	19.4 ^{abc}	22.7 ^{a}	22.6 ^{ab}	0.98	<0.001
	Leg muscles	14.1 ^{c}	16.7 ^{c}	16.9 ^{bc}	20.2 ^{ab}	20.7 ^{a}	1.02	<0.001
Drip loss	Pectorals	17.7 ^{a}	17.6 ^{a}	14.0 ^{b}	11.6 ^{b}	11.4 ^{b}	0.79	<0.001
	Leg muscles	18.3 ^{a}	17.6 ^{ab}	18.6 ^{a}	15.0 ^{bc}	14.7 ^{c}	0.88	<0.001

The data are expressed as means. Superscripts (a–c) with no common letters within a row show values that differ significantly ($p < 0.05$). SEM: Standard error of the mean.

3.4. Effects of Inorganic Zinc Supplementation in Low-Protein Diets on Serum Biochemical Indexes of Broilers

Table 7 describes the results of the serum biochemical assays conducted on the 42-day-old broilers. Upon analysis, it was found that the serum concentrations of ALP, TP, and GLO, as well as the A/G, did not show any significant variations across the different groups ($p > 0.05$). However, a noteworthy observation was the elevated level of ALB in the group that received 150 mg/kg of inorganic zinc, which was significantly higher than the other groups ($p < 0.05$). This enhancement, interestingly, was not statistically distinguishable when compared with the 130 mg/kg group. In light of these findings, it can be inferred that the strategic incorporation of inorganic zinc at concentrations of either 130 mg/kg or 150 mg/kg in low-protein diets has a distinct impact on serum biochemical indices of the broilers, indicating the potential benefits of zinc supplementation in poultry nutrition.

Table 7. Effects of gradual addition of inorganic zinc in the low-protein diet on serum biochemical indices of 42-day-old broilers.

Items	Content of Inorganic Zinc in a Low-Protein Diet					SEM	*p*-Value
	70 mg/kg (*n* = 6)	90 mg/kg (*n* = 6)	110 mg/kg (*n* = 6)	130 mg/kg (*n* = 6)	150 mg/kg (*n* = 6)		
ALP	2038.87	2270.70	2245.22	2308.92	2061.53	182.626	0.887
TP	23.47	23.55	24.33	26.62	24.77	1.481	0.063
ALB	10.80 ^{c}	11.05 ^{bc}	11.38 ^{abc}	12.43 ^{a}	12.20 ^{ab}	0.362	0.004
GLO	12.75	12.93	13.03	13.96	12.83	0.843	0.385
A/G	0.85	0.87	0.89	0.90	0.96	0.044	0.305

The data are expressed as means. Superscripts (a–c) with no common letters within a row show values that differ significantly ($p < 0.05$). Abbreviations: ALP, alkaline phosphatase; TP, total protein; ALB, albumin; GLO, globulin; A/G = ALP/GLO; SEM: Standard error of the mean.

3.5. Effect of Inorganic Zinc Supplementation in Low-Protein Diets on Intestinal Tissue Morphology of Broilers

The intestinal tissue morphology of the broilers was analyzed at 42 days of age; the results are illustrated in Figure 1. It was observed that the villus height in the group receiving 70 mg/kg of zinc was significantly lower than that of the other treatment groups. Furthermore, a significant increase in villus height was associated with increasing levels of zinc content in the low-protein diets ($p < 0.05$). In terms of crypt depth, no significant differences were detected between the 130 mg/kg and 150 mg/kg groups, as well as the 110 mg/kg group; however, these were notably lower than those observed in the 70 mg/kg group ($p < 0.05$). Regarding the ratio of villus height to crypt depth, the 130 mg/kg and 150 mg/kg groups demonstrated no significant disparities but exhibited considerably higher values in comparison to the other treatment groups ($p < 0.001$). Based on these results, it can be deduced that enhancing the level of inorganic zinc in the diet correlates with a progressive improvement in the intestinal development of broilers, a trend that was particularly prominent in the 130 mg/kg and 150 mg/kg groups.

At 42 days of age, fecal zinc concentrations illustrated a progressive decline in zinc excretion by broilers, concomitant with an increase in dietary zinc content, as depicted in Figure 2. Noteworthy is the observation that there were no significant differences in fecal zinc excretion levels between the 130 mg/kg and 150 mg/kg supplementation groups; however, the excretion levels in these two groups were markedly lower than those in the other treatment groups ($p < 0.001$). These findings suggest that the incorporation of 130 mg/kg or 150 mg/kg of inorganic zinc into the diet optimizes intestinal zinc absorption, leading to a reduction in environmental zinc discharge, and consequently, exhibits a more eco-friendly profile.

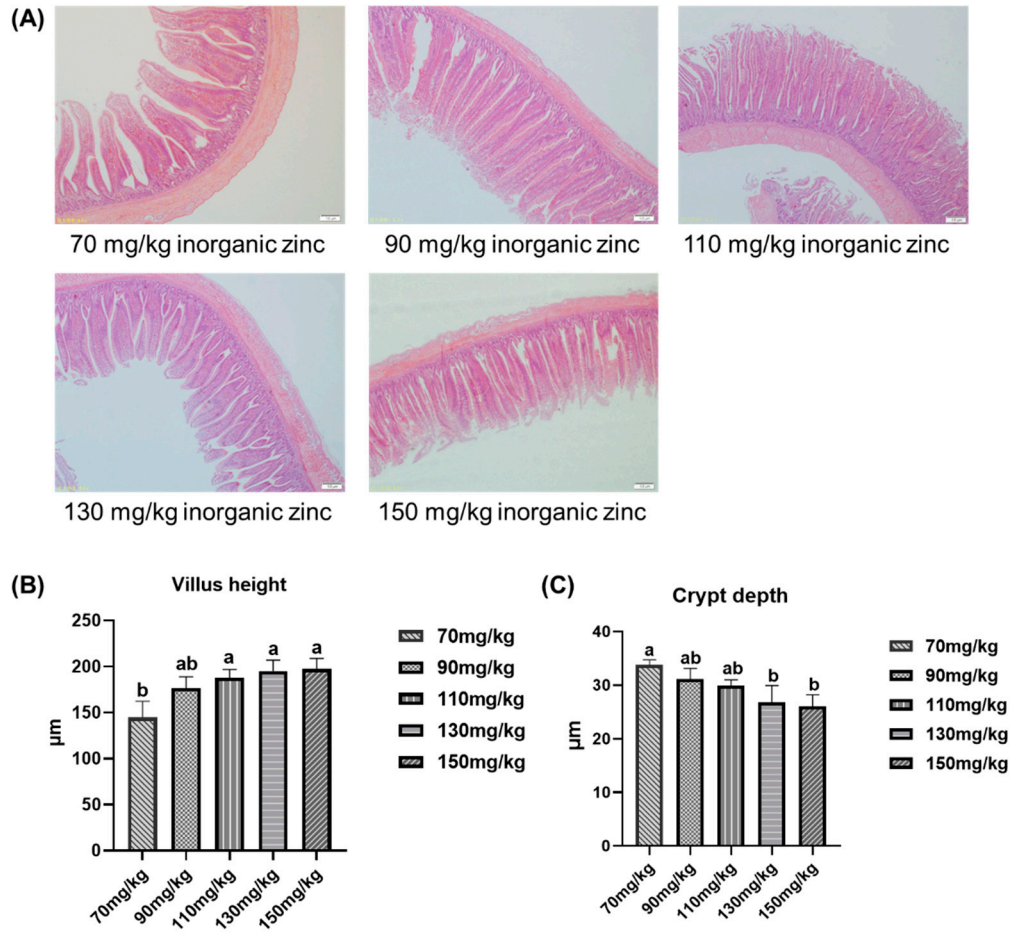

Figure 1. *Cont.*

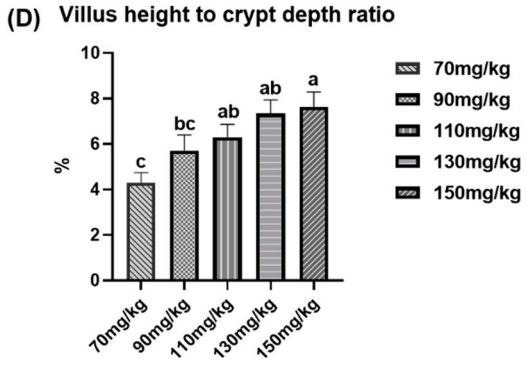

Figure 1. Effect of gradual inorganic zinc addition to low-protein diets on jejunal morphology of 42-day-old broilers. (**A**) The jejunal morphology of 42-day-old broilers with different levels of inorganic zinc supplementation ranging from 70 mg/kg to 150 mg/kg in low-protein diets; (**B**–**D**) Villus height, crypt depth, and villus height to crypt depth ratio of 42-day-old broilers with different levels of inorganic zinc supplementation ranging from 70 mg/kg to 150 mg/kg in low-protein diet. Superscripts (a–c) with no common letters within a row show values that differ significantly ($p < 0.001$).

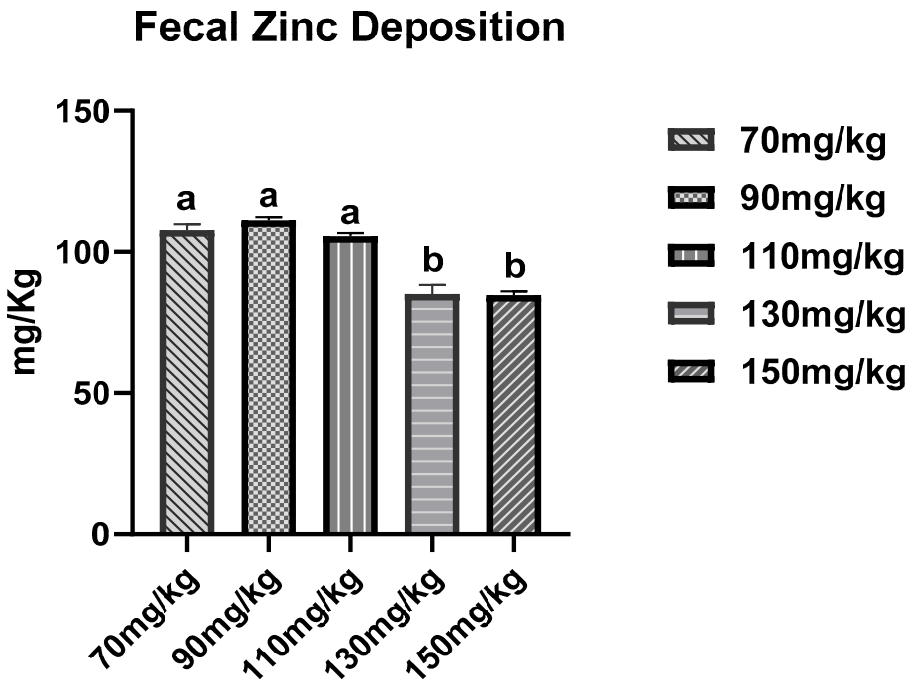

Figure 2. Effect of gradual addition of inorganic zinc to low-protein diets on zinc content in the manure of 42-day-old broilers. Superscripts (a,b) with no common letters within a row show values that differ significantly ($p < 0.001$).

3.6. Effects of Inorganic Zinc Supplementation in Low-Protein Diets on Gene Expression in Gut, Liver, and Muscles of Broilers

This study elucidates the impact of dietary inorganic zinc supplementation on the mRNA expression levels of various genes in broilers. Specifically, the MT gene in the liver exhibited significantly elevated expression levels in the 130 mg/kg and 150 mg/kg inorganic zinc groups compared with the other groups, as illustrated in Figure 3 ($p < 0.001$). A positive correlation was observed between the dietary levels of inorganic zinc and the mRNA expression of genes associated with intestinal tight junction proteins, including Occludin-1, Claudin-1, and ZO-1. Although the expression levels between the 130 mg/kg and 150 mg/kg groups were not statistically different, they were markedly higher than those in the other treatment groups ($p < 0.001$). Additionally, the genes associated with

muscle development, including MyoD, Myf5, and Myf6, exhibited significantly higher expression levels in the pectoral muscle of the broilers supplemented with 130 mg/kg and 150 mg/kg of inorganic zinc compared with the other three groups ($p < 0.001$). Furthermore, MyoG gene expression in the 150 mg/kg group was significantly upregulated compared with all other groups ($p < 0.001$), as presented in Figure 4. Drawing from these gene expression findings, it can be inferred that dietary supplementation with 130 mg/kg and 150 mg/kg of inorganic zinc positively influences the intestinal and muscular development of broilers whilst also enhancing the antioxidant capacity of their bodies, showing the multifaceted benefits of zinc supplementation in poultry nutrition.

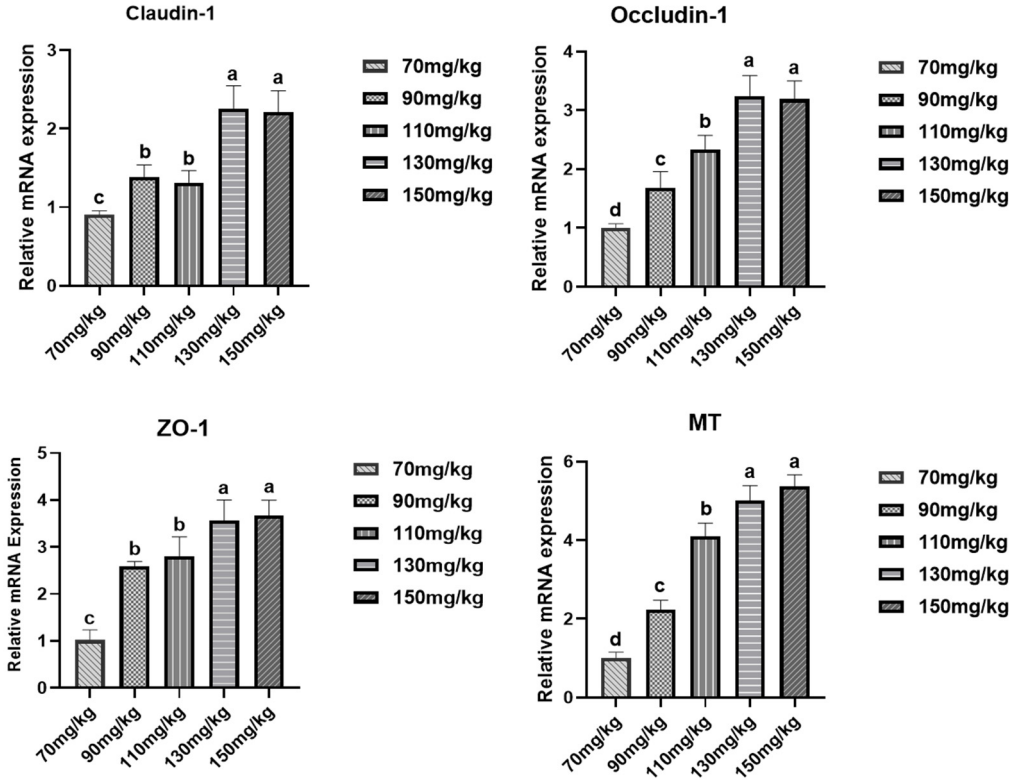

Figure 3. Effects of gradual addition of inorganic zinc to low-protein diets on intestinal transporter and liver MT activity in 42-day-old broilers. Superscripts (a–d) with no common letters within a row show values that differ significantly ($p < 0.001$).

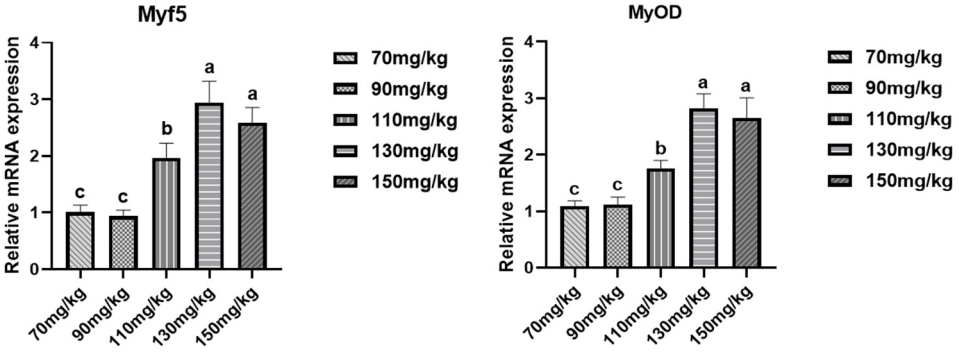

Figure 4. *Cont.*

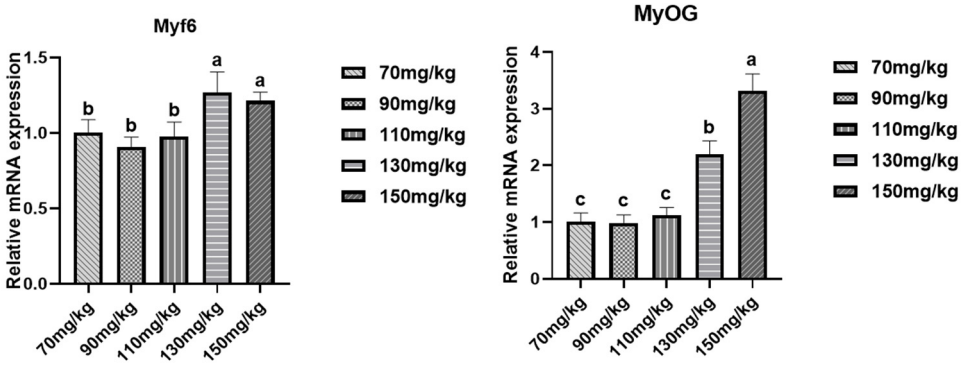

Figure 4. Effect of gradual addition of inorganic zinc to low-protein diets on the activity of the family of muscle regulators on 42-day-old broilers. Superscripts (a–c) with no common letters within a row show values that differ significantly ($p < 0.001$).

4. Discussion

Growth performance is a pivotal indicator of development and growth in livestock and poultry. The implementation of low-protein diets in animal feed has proven to be an effective strategy for conserving protein resources, enhancing protein utilization, reducing feed costs, and contributing to environmental sustainability. However, a direct reduction in dietary protein levels may potentially exert adverse effects on the growth performance of broilers [26]. To mitigate these effects, the supplementation of additives, including amino acids, alternative feed ingredients, and enzymes, in low-protein diets can act as a compensatory mechanism, counterbalancing the decline in growth performance induced by the reduced protein content.

Zinc, a vital trace mineral in animal physiology, is commonly incorporated into broiler feed in both organic and inorganic forms. It plays an integral role in promoting animal growth and skeletal muscle development [27]. Variations in dietary feed intake are observed despite equivalent effects on growth performance between different forms of zinc [28]. A deficiency of zinc in the diet can hinder the assimilation of certain amino acids, diminish protein synthesis, and ultimately impede the growth performance of broilers [29]. Enriching broiler feeds with zinc not only enhances growth performance but also increases the zinc content in tissues and feces and improves meat quality [30]. Numerous studies have reported a lack of significant influence of zinc supplementation on the carcass traits of broilers [31]. However, our current study reveals a notable impact on both the total evisceration rate and the pectoral muscle ratio when broiler feed is supplemented with 130 mg/kg or 150 mg/kg of inorganic zinc. This discrepancy could stem from the insufficient zinc concentrations applied in previous studies. Elevated abdominal fat rates can negatively affect both feed conversion ratios and meat quality in broilers [32], highlighting the necessity to reduce abdominal fat content. Zinc can modulate carcass quality by influencing fat composition, as observed in our study, where a significant decreasing trend was noted in abdominal fat rates in the 130 mg/kg and 150 mg/kg supplementation groups. Key indicators for evaluating the quality of livestock and poultry meat include muscle color (L*), pH, drip loss, shear force, and hydration. Dietary zinc has proven effective in enhancing the quality of broiler breast muscles. However, some studies suggest that increasing dietary zinc does not substantially affect broiler meat quality [33]. In addition, the inclusion of 120 mg/kg of zinc in the feed does not significantly alter the tenderness and color of broiler breasts [34]. Our study did not find significant enhancements in the meat quality of broiler chickens until the inorganic zinc content was increased to 130 mg/kg or 150 mg/kg, highlighting the potential influence of dietary zinc content, broiler breed, and rearing environment.

Alterations in serum biochemical indices offer valuable insights into the zinc nutrient absorption and metabolic capacities of the organism, while simultaneously providing indicators of growth and development. Dietary zinc deficiency may lead to decreased levels

of ALB and GLO, whereas the addition of an optimal amount of zinc has the potential to elevate these levels. The zinc concentration in serum reflects the organism's capacity for zinc storage and, when bound to ALB, forms zinc protein, indicating, to some extent, the body's proficiency in absorbing serum protein nutrients. Zinc, as a metal ion, is integral to the synthesis of ALP, functioning as both a component and an activator of the enzyme's active center, and exhibits a positive correlation with enzyme activity. Previous research has highlighted the significant impact of dietary zinc on ALP activity in rat serum [35], and a corresponding increase in ALP activity in broiler serum with dietary zinc supplementation [6]. A previous study demonstrated the efficacy of zinc supplementation in improving growth performance, reproductive capabilities, zinc concentration, and antioxidant capacity in broilers [36]. In line with these findings, our current investigation ascertains that the inclusion of 130 mg/kg of inorganic zinc during the low-protein feeding phase markedly influences the serum biochemical indices of 42-day-old broilers. While no significant differences were observed in the 150 mg/kg group, a declining trend was apparent.

The intestine not only functions as the primary site for nutrient absorption in animals but also provides a crucial level of defense against exogenous bacteria and viruses, playing a vital role in maintaining overall health. Consequently, intestinal damage can lead to a decline in production performance [15]. The intact morphological structure of the jejunum serves as a valuable indicator of intestinal health; an increase in the surface area of the intestinal villi is indicative of robust nutrient absorption capabilities. Existing research supports the notion that supplementing low-protein diets with additives can enhance the development and health of the intestinal barrier [37]. Other studies have demonstrated that including 120 mg/kg of zinc in broiler feed can promote the growth of jejunal villi [38]. Additionally, the administration of high levels of zinc has been associated with an accelerated rate of crypt cell maturation in the third small intestine segment of mice [16]. This suggests that dietary zinc supplementation can expedite the maturation of crypt cells, contributing to a stable small intestine mucosal barrier. Such stability enhances resistance against exogenous pathogenic bacteria, improves the intestine's nutrient absorption and metabolism, and consequently leads to overall performance enhancements in animals. Our current findings substantiate that supplementing with 130 mg/kg or 150 mg/kg of inorganic zinc during periods of low protein intake significantly influences the intestinal morphological parameters of 42-day-old broilers, underscoring the beneficial impact of zinc on intestinal health and function.

The over-excretion of trace elements during livestock and poultry farming is a principal contributor to environmental pollution. To accurately determine the optimal amount of trace elements to include in feed, it is crucial to assess the bioavailability of zinc in feedstuffs, specifically the extent to which it is excreted from the body. Current research indicates that the concentration of zinc in broiler manure increases proportionally with dietary zinc levels [39,40], a phenomenon potentially attributed to suboptimal absorption efficiency within the organism. However, given that excretion encompasses both feces and urine, the quantity of zinc excreted does not solely reflect the amount of zinc deposited in the body. Our current study has elucidated that, when the zinc content in the low-protein diet was increased to 130 mg/kg, there was a noticeable decrease in zinc excretion. This implies that a diet including 130 mg/kg of inorganic zinc is more efficiently absorbed by broiler chickens, leading to a reduction in zinc excretion and, consequently, mitigating environmental pollution. This finding is critical for optimizing zinc supplementation strategies in poultry diets aiming to enhance absorption efficiency, promote animal health, and reduce the environmental footprint of poultry production.

Occludin, Claudin-1, and ZO-1 are pivotal components that constitute and regulate the structure and function of intestinal tight junctions in animals. The intermolecular interaction between Occludin and Claudin proteins is essential for maintaining the integrity and stability of intestinal tight junctions. Meanwhile, the expression level of the ZO-1 protein serves as a vital marker for modulating intestinal permeability, with all three proteins collectively orchestrating the dynamic regulation of tight junction structures

and functions [41]. Previous research has documented that high-zinc diets significantly enhance the mRNA expression levels of ZO-1 and Occludin in the gastrointestinal tract of early-weaned piglets [42]. Additionally, elevated zinc levels have been associated with an increase in the expression of jejunal tight junction protein-1 and Claudin-1 in broilers [43]. These findings emphasize the potential of dietary zinc supplementation in preserving the integrity of the intestinal mucosa, thereby offering protection against damage inflicted by pathogenic bacteria on the animal's intestinal tract. Our results suggest that administering feed supplemented with inorganic zinc at concentrations ranging from 130 to 150 mg/kg during the low-protein feeding stage had a profound impact on the gene expression of intestinal tight junction proteins in 42-day-old broilers.

Zinc ions play a crucial role in regulating the MT gene within the body, exerting control over the augmentation of MT at the transcriptional level. MT-1 is predominantly expressed in the livers of animals, and a robust positive correlation has been established between MT mRNA concentrations and dietary zinc intake [44]. In instances of zinc deficiency, there is a marked decrease in MT gene expression, leading to reduced MT–zinc binding and consequently preventing a sharp decline in plasma zinc concentrations. On the other hand, under conditions of elevated zinc availability, MT gene expression is upregulated, facilitating the binding of large quantities of zinc. This mechanism acts as a protective buffer, inhibiting excessive plasma zinc concentrations and mitigating the risk of zinc toxicity. This regulatory dynamic also indicates that the expression of MT, a zinc-binding protein, is upregulated in response to mechanisms related to oxidative stress [45]. Such findings lend substantial support to the concept that zinc plays an integral role in maintaining bodily homeostasis and proper physiological function through the modulation of MT gene expression. We observed that the inclusion of 150 mg/kg of inorganic zinc in the diet during a period of low protein intake exerted a significant influence on the expression of MT in the livers of 42-day-old broilers, highlighting the intricate relationship between dietary zinc, gene expression, and overall hepatic function.

The myogenic regulatory factor (MRF) family comprises four pivotal transcription factors: MyoD, MyoG, Myf5, and Myf6. Each member of the MRF family is instrumental in orchestrating skeletal muscle development in animals, propelling the proliferation and differentiation of muscle cells [46]. There is an existing body of research suggesting that broilers can potentially expedite the differentiation of myogenic genes by enhancing the transcriptional abundance of Myf5, Myf6, and MyoG mRNA [47]. Consistent with these findings, our study reveals that supplementation of broiler feed with inorganic zinc at concentrations ranging from 130 to 150 mg/kg during a low protein intake phase substantially amplifies the transcription of muscle-related genes in 42-day-old broilers. This underlines the vital influence of dietary zinc on muscle gene expression and development in broilers.

The endogenous concentration of zinc in feed is typically suboptimal, necessitating the prevalent practice of zinc fortification to achieve adequate nutritional levels. This augmentation is conducted within a substantial safety margin to mitigate potential risks. Accordingly, this study adheres to the guidelines established by the European Commission, maintaining the total zinc content within a range of 70–140 mg/kg [22]. This strategic modulation of zinc concentrations in the feed aims to elucidate its impact on broilers. It is crucial to highlight that the zinc values reported in the present study represent the total zinc content, ensuring direct and practical relevance to poultry production scenarios. This approach stands in contrast to the predominant focus on supplemental zinc values in the existing literature. By adopting this comprehensive perspective on total zinc content, this study contributes to a more holistic understanding of zinc's role in poultry nutrition, with potential implications for optimizing poultry production practices while adhering to safety standards.

5. Conclusions

Based on this study, we conclude that the supplementation of low-protein diets with inorganic zinc significantly enhances muscle development and zinc absorption in broilers. This approach also results in the refinement of intestinal morphology and modulates gene expression relevant to both intestinal and muscle development. Moreover, it proves effective in regulating fecal zinc excretion. These findings indicate the crucial role of strategic dietary zinc supplementation in fostering broiler health and productivity, thereby contributing substantially to sustainable advancement in poultry farming practices. This not only aligns with industry standards but also opens avenues for further research and implementation of nutritional strategies aimed at optimizing poultry performance and welfare.

Author Contributions: D.P.: Conceptualization, Writing—original draft, Writing—review and editing; Y.J.: Writing—review and editing, Project administration; C.Z.: Conceptualization, Writing—review and editing, Supervision, Funding acquisition; R.S.: Formal analysis, Data curation, Writing—original draft; Y.L.: Writing—review and Project administration; Y.H.: Investigation, Resources; H.C.: Investigation, Resources; Y.Z.: Writing—review and editing; J.Z.: Writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The animal study protocol was approved by the the Animal Ethics and Welfare Committee of Jilin University (protocol code SY202107300 and date of approval:20210709).

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest: Co-first author Yougang Jia has been involved with New Hope Liuhe Company, while author Changhai Zhou has received research grants from the Key Laboratory of Quality Control for Feed and Livestock and Poultry Products in Sichuan Province; the remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Abbreviations

AA, Arbor Acres; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Myf5, myogenic factor 5; Myf6, myogenic factor 6; MyOD, myogenic differentiation 1/myoblast determination protein 1; MyOG, Myogenin; MT, Metallothionein; ZO-1, zonula occludens-1; FCR, feed conversion ratio.

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