Effects of rare earth-chitosan chelate on growth performance, antioxidative
and immune function in broilers

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ABSTRACT
This research aims to study the effects of rare earth-chitosan chelates (RECC) on growth performance, immunity and antioxidant function in broilers. A total of 192 one-day-old mixed-sex Arbour Acres (AA) broilers were allotted into 4 treatment groups with 6 replicates per treatment and 8 broilers per pen. Broilers were fed the basal diet supplemented with, respectively, 0 (control group), 150, 200 and 250 mg/kg RECC for 42 d. The results showed that dietary RECC improved average daily gain and decreased feed/gain \(p < .05\). Broilers fed with appropriate dose of RECC increased total antioxidant capacity (T-AOC) in serum (d 42) and liver (d 21). And the activity of glutathione peroxidase (GSH-Px), catalase (CAT) and superoxide dismutase (SOD) were increased, whereas malonaldehyde (MDA) concentration was decreased on d 42 in liver of broilers \(p < .05\) by enhancing the related gene expression in nuclear factor erythroid 2-related factor 2 (Nrf2) pathway. In addition, supplementing appropriate dose of RECC in diet increased the concentrations of interleukin 2 (IL-2), IL-4 in serum and jejunum (d 42). And the concentrations of immunoglobulin M (IgM) (d 21), and secretory immunoglobulin A (sIgA), IgG (d 42) \(p < .05\) in jejunum were elevated by decreased the related gene expression in Toll-like receptor 4/nuclear factor kappa-B (TLR4/NF-\(\kappa\)B) pathway. In conclusion, RECC could exert beneficial effects on growth performance, immune function and antioxidant capacity of broilers, and the optimal supplemental dose for broiler production is 175–200 mg/kg.

HIGHLIGHTS
• This paper was very important in revealing the action mechanism of rare earth-chitosan chelate on immune function and antioxidant capacity of animals.
• The results of this study will provide a theoretical basis for further developing rare earth-chitosan chelate as antibiotic alternatives.
• The optimal dose range of rare earth-chitosan chelate in diet of broilers was 175–200 mg/kg.

Introduction
In recent decades, poultry meat has become one of the most important sources of animal protein in daily life due to its high production efficiency, low cost, no religious restriction and variety of consumption options (Kleyn and Ciacciariello 2021). However, the adverse effects of intensive breeding environment can lead to immune and oxidative stress in broiler chickens. The pathogens take advantage of this situation to increase the infection rate of the disease, lead to imbalance between free radicals and antioxidant systems, caused tissue damage, and affected the growth of broilers (Xi and Dong 2019; Zhang et al. 2019; Abo Ghanima et al. 2020). Therefore, improving immune function and antioxidant capacity of broilers through nutritional regulation was a potential way to improve growth performance and the health of broilers.

Rare earth element (REE) consists of 14 natural elements. It is widely distributed in China, the United States, India, Russia and other countries. As a new and safe feed additive, REE has been widely used in livestock and poultry production. A large number of studies have found that adding REE can improve animal weight gain, milk yield, egg production capacity and feed conversion efficiency to varying degree. (Cai et al
2018; Tariq et al. 2019). Previous studies have shown that rare earth elements (such as Ce and La) could regulate the immune function and antioxidant capacity of animals, promote intestinal nutrient absorption and increase the synthesis capacity of microbial protein (Abdelnour et al. 2019; Adeel et al. 2019). Through research of recent years, it is found that dietary Ce and La could not only effectively balance the homeostasis of oxidation system in the blood and liver (Nikitchenko et al. 2021), but also improve the nutrient digestibility and growth performance of animals (Cai et al. 2018). Chitosan is the second most abundant renewable biopolymer in the world, which has been widely used as a feed additive in recent years. Studies have found that chitosan can improve the intestinal villus morphology and promote the absorption of nutrients to improve the growth performance of animals, inhibit the colonisation of harmful bacteria such as Escherichia coli and Salmonella, and improve the immune function of the body. In addition, chitosan can also be used as an antioxidant in animal production due to its excellent free radical scavenging ability (Ma et al. 2017; Yuan et al. 2019; Pereira et al. 2020). As a potential feed additive, rare earth-chitosan chelate (RECC) was prepared by wrapping rare earth with chitosan as dilution carrier and special processing, which could improve the growth performance and immunity of fish (Zhou et al. 2016). However, as far as we know, studies on the effects of RECC as a feed additive on growth performance and health of poultry are limited, especially in broiler’s antioxidant capacity and immune function. The potential action mechanisms of RECC in broilers need to be further explored. Therefore, this experiment was conducted to investigate the effects of RECC supplementation on growth performance, antioxidative and immune function of broilers, and find the appropriate supplemental level of RECC in broiler diets, so as to provide theoretical basis for its application in poultry production.

Materials and methods

Experimental animals and management

RECC was provided by Shenzhen Xikean Industrial Co., Ltd. (Shenzhen, China), in which the content of rare earth salt is not less than 32%, and the active components of rare earth are cerium and lanthanum. A total of 192 one-day-old mixed-sex Arbou Acres (AA) broilers were obtained from a local hatchery, and were randomly divided into 4 groups with 6 replicates of 8 birds each. There was no significant difference in initial body weight among all groups ($p > .05$).

Birds were fed the basal diet supplemented with, respectively, 0 (control group), 150, 200, 250 mg RECC per kg of diet. The basal diet was formulated based on the nutrient recommendations of Feeding Standard of Chicken, China (NY/T 33-2004) (Chinese Ministry of Agriculture 2004) (Table 1). Feed and water were available ad libitum for broilers during the experiment.

Housing

Chicks were weighed and placed in cages (100 × 50 cm$^2$). The room temperature was set at 33 °C in the first week, slowly decreased by 3 °C every week, and remained stable when reaching 21 °C. The humidity in the house was maintained at 50%–60%. Birds were vaccinated for Newcastle disease, infectious bronchitis and infectious bursal disease. All experimental procedures were carried out in accordance with the national standard Guideline for Ethical Review of Animal Welfare (GB/T 35892-2018).

Growth performance measurement

At the age of 1, 21 and 42 d, body weight and total feed intakes in each replicate were recorded. The average daily feed intake (ADFI), average daily

| Table 1. Composition and nutrient levels of basal diets (air-dry basis)%.
| Items | 1 to 21 days of age | 22 to 42 days of age |
|-------|---------------------|---------------------|
| Ingredients | | |
| Corn | 52.50 | 58.80 |
| Soybean meal | 40.00 | 33.80 |
| Soybean oil | 3.00 | 3.00 |
| Dicalcium phosphate | 1.90 | 1.80 |
| Limestone | 1.08 | 1.22 |
| Salt | 0.37 | 0.37 |
| L- Lysine | 0.05 | 0.03 |
| DL- Methionine | 0.19 | 0.07 |
| Premix$^a$ | 0.80 | 0.80 |
| Choline | 0.11 | 0.11 |
| Total | 100.0 | 100.0 |
| Nutrient levels$^b$ | | |
| Metabolizable energy/(MJ/kg) | 12.42 | 12.62 |
| Crude Protein | 21.77 | 19.65 |
| Calcium | 1.00 | 1.02 |
| Available Phosphorus | 0.44 | 0.42 |
| Lysine | 1.34 | 1.15 |
| Methionine | 0.55 | 0.40 |
| Cystine | 0.40 | 0.36 |
| Methionine + Cystine | 0.95 | 0.76 |
| Threonine | 0.40 | 0.38 |
| Tryptophan | 1.02 | 0.87 |

$^a$Vitamin premix provided the following per kilogram of diet: vitamin A 9000 IU, vitamin D3 3,000 IU, vitamin E 26 mg, vitamin K3 1.20 mg, vitamin B1 0.012 mg, vitamin B2 8.00 mg, vitamin B6 4.40 mg, vitamin B12 0.012 mg, niacin 45 mg, folic acid 0.75 mg, biotin 0.20 mg, choline 1100 mg, choline pantothenate 15 mg, Fe 100 mg, Cu 10 mg, Zn108 mg, Mn 120 mg, I 1.5 mg, Se 0.35 mg.

$^b$Crude protein was measured value, while others were all calculated values.
gain (ADG) and feed/gain ratio (F/G) were calculated for each period.

Sample collection
At 21 and 42 days old, after a period of 24-h fasting, one broiler was randomly selected in each replicate to collect 10 mL of blood from the wing vein and placed in a non-heparin sodium tube. After standing at room temperature for 45 minutes, the blood was centrifuged at 3000 x g for 15 minutes, and the upper serum was collected and stored at −20°C for further analysis (Guo et al. 2020). After that, the chickens were killed by cervical dislocation. The liver and jejunum were carefully removed and placed on ice. The sample from the right lobe of the liver was wrapped in the prepared tinfoil, and the sample from the left lobe of the liver was placed in the cryogenic vials; the chyme in jejunum was rinsed with ice-cold normal saline, and the intestine segment chosen about 5 cm was wrapped in foil paper. The jejunum mucosa was scraped with a clean blade and put into cryogenic vials. The samples packed in tinfoil paper were frozen at −20°C, and the cryogenic vials were frozen quickly in liquid nitrogen and stored at −80°C for future index measurement.

Preparation of liver and jejunum homogenate
About 0.5 g samples of liver tissue and jejunum mucosa were weighed and homogenised in 0.9% ice-cold isotonic sodium chloride solution with a handheld homogeniser (FA6/10, FLUKO, Shanghai) at the ratio of 1:9 (wt/vol). The homogenate was centrifuged at 4 °C for 15 min at 3000 x g, and the supernatant was taken and stored at −80 °C. The protein concentration in the supernatant of liver homogenate was determined by the coomassie bright blue method with commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the specifications.

Determination of immune indices in serum and jejunum
The contents of interleukin-1β, IL-2, IL-4, IL-6 and secretory immunoglobulin (sIg) A, IgM and IgG in serum and jejunum mucosa homogenate were determined by chicken-specific ELISA kits (Quanzhou Ruixin Biotechnology Co., LTD.) according to manufacturer’s instructions.

Determination of antioxidant indices in serum and liver
Total antioxidant capacity (T-AOC), malondialdehyde (MDA) content, superoxide dismutase (SOD) activity, glutathione peroxidase (GSH-Px) activity and catalase (CAT) activity in serum and tissue homogenate were determined with commercial antioxidant kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the specifications.

Real-time PCR
Total RNA was extracted from liver and jejunum tissues with RNAiso Plus reagent (TaKaRa) according to the instructions. The total RNA was quantitatively and qualitatively determined at 260 nm and 280 nm using a nucleic acid quantifier. Total RNA was reverse-transcribed into cDNA by Hifair® 1st Strand kit (Yeasen Biotech), and real-time quantitative PCR was performed using Roche LightCycler® 96 qPCR tester and Hieff® qPCR SYBR Green Master Mix kit. Each sample had 2 replicates. The sequences of target genes and primers were shown in Table 2, which were designed and synthesised by Shanghai Sangon Biotech (Shanghai, China). β-actin was used as a reference gene and the relative expression levels of different treatment groups were calculated by 2−ΔΔCT (Guo et al. 2020).

Statistical analysis
All data were preliminarily processed by Excel 2010. SAS 9.2 statistical software was used for statistical analysis with repetition as the experimental unit. The linear and quadratic effects of RECC supplemental level on growth performance, immune and antioxidant function of broilers were investigated by regression analysis. p < .05 indicates that the data was statistically significant.

Results
Growth performance
The effects of dietary RECC on the growth performance (ADG, ADFI and F/G) in broilers were shown in Table 3. Regression analysis showed that, with the increase of RECC supplemental level, ADG showed a significant linear or quadratic effect from d 1 to 21 (p < .05), and a significant quadratic effect (p < .05) from d 22 to 42; F/G radio showed a significant linear or quadratic reduction during d 1–21 and d 22–42 (p < .05). From day 1 to 42, ADG had a significant
linear or quadratic increase ($p < .05$), and F/G showed a significant linear ($p < .05$) or extremely significant quadratic ($p < .01$) reducing effect. There was no significant change in ADFI during the experiment.

Compared with the control group, RECC supplementation at 150 and 200 mg/kg significantly increased ADG and decreased F/G in broilers during d 22 to 42 and d 1 to 42. The best supplemental doses for broilers were 175 mg/kg for ADG and 200 mg/kg for F/G, which were calculated by using the regression equation: $Y = -9E-05x^2 + 0.0315x + 57.249$ and $Y = 2E-06x^2 - 0.0008x + 1.5708$.

### Antioxidant index

The effects of dietary RECC on the serum antioxidant capacity of broilers were shown in Table 4. On d 42 of the experiment, with the increase of RECC supplemental dose, the serum T-AOC content showed a significant quadratic increase effect ($p < .05$). Compared with the control group, 200 mg/kg supplementation increased the activity of T-AOC and GSH-Px in serum on d 21 and 42, whereas decreased the content of MDA in serum on d 42.

The effects of dietary RECC on the liver antioxidant capacity of broilers were shown in Table 5. On d 21 of the experiment, with the increase of RECC supplemental level, T-AOC presented an extremely significant linear or quadratic enhancement effect ($p < .01$), and the content of MDA linearly reduced ($p < .05$). On d 42, the activity of CAT, T-SOD and GSH-Px showed a significant quadratic increasing effect ($p < .05$), and MDA in liver showed an extremely significant linear or quadratic reducing effect with the increase of RECC.

### Table 2. Primer sequence of target and reference genes.

| Gene name | Primer sequences (5' to 3') | GenBank accession No. | Length |
|-----------|-----------------------------|-----------------------|--------|
| β-Actin   | F: GCCAACGAGAGAAGATGACAC    | NM_205518             | 118 bp |
|           | R: GTAAACACCTCACACAGGTCAC  |                       |        |
| TLR4      | F: TTTGACCGACCTCTTGAGTG    | NM_001030693          | 131 bp |
|           | R: CACAGCAGATGTTGACGTTG    |                       |        |
| MyD88     | F: CACGGCTGCTGCCTCAGCA     | NM_001030962          | 198 bp |
|           | R: CACGCCAGAACCCAAACCTCT  |                       |        |
| NF-κB p65 | F: CAGCCCATCTATGACAAAAGC   | D13721                | 151 bp |
|           | R: CAGCCCAAGCCAAACCTCTCA  |                       |        |
| NF-κB p50 | F: CAGCCCAAGCCAAACCTCTCA  | NM_205134             | 80 bp  |
|           | R: CAGCCCAAGCCAAACCTCTCA  |                       |        |
| IL-6      | R: CCGCTCAGGGCTTTGAGTAC    | HM179640              | 106 bp |
| SOD       | F: TGTGCAAGAAGATGCTTCTGC   | NM_2050641            | 98 bp  |
|           | R: TGCTGGCTCCAGGTTAAAGTG   |                       |        |
| CAT       | F: GTGCAAGAAGATGCTTCTGC    | NM_001031215.1        | 182 bp |
| GPx7      | F: CAAAGTTGCGGTCAGTGGA     | NM_001163245.1        | 136 bp |
| Nrf2      | R: GATGTCACCCTGCCITTTAG    | NM_205117.1           | 215 bp |
|           | C: CTGCCACCATGTATTCTC      |                       |        |

TLR4: Toll-like receptor 4; MyD88: myeloid differentiation factor 88; NF-κB p65: nuclear factor kappa-B p65; NF-κB p50: nuclear factor kappa-B p50; IL-6: Interleukin-6; SOD: total superoxide dismutase; CAT: catalase; GPx7: glutathione peroxidase 7; Nrf2: nuclear factor erythroid 2-related factor; F: forward primer; R: reverse primer.

### Table 3. Effects of RECC on the growth performance in broilers.

| Items          | Dietary RECC level (mg/kg) | SEM  | Linear | Quadratic |
|----------------|---------------------------|------|--------|-----------|
|                | 0 (CON)                   | 150  | 250    |           |
| ADG g/d        |                           |      |        |           |
| d1–21          | 30.27b                    | 30.80ab | 31.68a | 31.39ab   | 0.009 | 0.012 | 0.047 |
| d22–42         | 84.17c                    | 87.96ab | 90.12a | 86.02ab   | 0.690 | 0.140 | 0.029 |
| d1–42          | 57.28b                    | 59.56a | 60.29a | 59.11a    | 0.348 | 0.023 | 0.016 |
| ADFI g/d       |                           |      |        |           |
| d1–21          | 39.99                      | 40.04 | 40.14  | 40.32     | 0.044 | 0.689 | 0.901 |
| d22–42         | 137.28                     | 139.31 | 141.15 | 140.42    | 1.650 | 0.163 | 0.376 |
| d1–42          | 85.52                      | 86.92 | 89.53  | 88.91     | 1.856 | 0.122 | 0.310 |
| F/G            |                           |      |        |           |
| d1–21          | 1.33a                      | 1.30ab | 1.27b  | 1.30ab    | 0.011 | 0.021 | 0.047 |
| d22–42         | 1.91b                      | 1.79b | 1.79b  | 1.78b     | 0.027 | 0.023 | 0.024 |
| d1–42          | 1.57a                      | 1.51b | 1.50b  | 1.52b     | 0.020 | 0.013 | 0.005 |

Means within the same row with no common superscript differ significantly ($p < .05$).

CON: control group; RECC: rare earth-chitosan chelates; ADG: average daily gain; ADFI: average daily feed intake; F/G: feed to gain ratio; SEM: standard error of means.

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[Table 2, Table 3 continued]
In addition, compared with the control group, RECC supplemental level of 200 and 250 mg/kg improved the antioxidant capacity of the liver.

**Antioxidant related gene expression levels**

The mRNA expression levels of antioxidant genes in liver were shown in Table 6. On d 42 of the experiment, the expression levels of Nrf2, CAT and GPX-7 showed a very significant quadratic increase effect with the increase of RECC supplemental dose (p < .01), and SOD expression showed a very significant linear (p < .01) or significant quadratic (p < .05) increasing effect. Compared with the control group, RECC supplementation increased the expression of antioxidant genes, but the mRNA gene expression showed a downward trend when the supplemental level was higher than 200 mg/kg.

**Immune index**

The effect of dietary RECC on serum immune indexes of broilers were shown in Table 7. On d 21 of the experiment, IgA showed a significant quadratic increasing effect (p < .05), and IL-1β, IL-6 showed an extremely significant linear decreasing effect (p < .01) with the increase of RECC dosage. On d 42, IgG, IL-2 and IL-4 showed a significant linear or quadratic increasing effect (p < .01). Compared with the control group, RECC supplementation decreased the contents

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**Table 4. Effects of RECC on the serum antioxidant indexes in broilers.**

| Items        | Dietary RECC level (mg/kg) | p-value     |
|--------------|----------------------------|-------------|
|              | 0 (CON)       | 150 | 200 | 250 | SEM | Linear | Quadratic |
| d 21 CAT, U/mL | 1.88 | 2.12 | 2.47 | 2.09 | 0.189 | 0.225 | 0.368 |
| SOD, U/mL    | 132.68 | 129.86 | 132.33 | 127.32 | 15.888 | 0.868 | 0.983 |
| GSH-Px, U/mL | 2018.81b | 1934.46b | 2230.40ab | 2038.40b | 49.886 | 0.478 | 0.619 |
| T-AOC, μmol/mL | 0.72ab | 0.77a,b | 0.81* | 0.75* | 0.014 | 0.109 | 0.066 |
| MDA, nmol/mL | 2.51 | 2.83 | 2.74 | 2.63 | 0.118 | 0.331 | 0.247 |
| d 42 CAT, U/mL | 2.06 | 2.36 | 1.87 | 2.57 | 0.194 | 0.316 | 0.497 |
| SOD, U/mL    | 367.00 | 328.85 | 348.26 | 311.76 | 19.503 | 0.186 | 0.430 |
| GSH-Px, U/mL | 1878.80 | 2216.10 | 2013.50 | 1998.50 | 146.489 | 0.661 | 0.591 |
| T-AOC, μmol/mL | 0.59ab | 0.66ab | 0.69* | 0.62ab | 0.019 | 0.140 | 0.030 |
| MDA, nmol/mL | 2.88a | 2.55ab | 2.13b | 2.99* | 0.144 | 0.890 | 0.078 |

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**Table 6. Effects of RECC on the gene expression of liver antioxidant related factors of broilers.**

| Items        | Dietary RECC level (mg/kg) | p-value     |
|--------------|----------------------------|-------------|
|              | 0 (CON)       | 150 | 200 | 250 | SEM | Linear | Quadratic |
| d 21 Nrf2    | 1.00 | 0.90 | 0.99 | 0.90 | 0.037 | 0.250 | 0.601 |
| CAT          | 1.00 | 1.05 | 1.03 | 1.16 | 0.043 | 0.248 | 0.424 |
| SOD          | 1.00 | 1.04 | 1.02 | 1.07 | 0.060 | 0.755 | 0.951 |
| GPx-7        | 1.00 | 1.04 | 1.16 | 1.09 | 0.045 | 0.295 | 0.583 |
| d 42 Nrf2    | 1.00b | 1.26a | 1.16ab | 1.05b | 0.031 | 0.233 | 0.003 |
| CAT          | 1.00b | 1.15* | 1.08ab | 1.06ab | 0.017 | 0.125 | 0.004 |
| SOD          | 1.00b | 1.08ab | 1.10b | 1.18* | 0.023 | 0.003 | 0.111 |
| GPx-7        | 1.00ab | 1.15* | 1.06a | 0.94b | 0.032 | 0.602 | 0.006 |

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**Table 7. Effects of RECC on the liver antioxidant indexes of broilers.**

| Items        | Dietary RECC level (mg/kg) | p-value     |
|--------------|----------------------------|-------------|
|              | 0 (CON)       | 150 | 200 | 250 | SEM | Linear | Quadratic |
| d 21 CAT, U/mg prot. | 5.15 | 6.27 | 5.36 | 6.52 | 0.603 | 0.232 | 0.493 |
| SOD, U/mg prot.     | 1110.50 | 1119.70 | 1129.90 | 1094.60 | 53.502 | 0.978 | 0.956 |
| GSH-Px, U/mg prot.  | 22.12 | 21.01 | 20.63 | 24.61 | 0.736 | 0.357 | 0.050 |
| T-AOC, μmol/g prot. | 62.84c | 65.24bc | 66.50b | 69.99a | 0.801 | 0.002 | 0.003 |
| MDA, nmol/g prot.   | 0.38 | 0.32 | 0.26 | 0.26 | 0.025 | 0.013 | 0.052 |
| d 42 CAT, U/mg prot. | 9.16b | 13.74a | 11.62ab | 11.159b | 0.745 | 0.224 | 0.023 |
| SOD, U/mg prot.     | 864.79b | 987.03ab | 1161.79a | 1021.96ab | 44.561 | 0.014 | 0.042 |
| GSH-Px, U/mg prot.  | 23.19bc | 26.91a | 20.63 | 21.12c | 0.598 | 0.717 | 0.002 |
| T-AOC, μmol/g prot. | 70.65 | 74.09 | 76.60 | 84.06 | 3.571 | 0.080 | 0.147 |
| MDA, nmol/g prot.   | 0.70b | 0.59a | 0.50b | 0.36b | 0.044 | 0.002 | 0.006 |

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Means within the same row with no common superscript differ significantly (p < .05).

CON: control group; RECC: rare earth-chitosan chelates; CAT: catalase; SOD: superoxide dismutase; GSH-Px: glutathione peroxidase; T-AOC: total antioxidant capacity; MDA: malondialdehyde; SEM: standard error of means.
of inflammatory cytokines IL-1β and IL-6 in serum on d 21, and 200 and 250 mg/kg RECC supplementation increased the contents of IgG, IL-2 and IL-4 on d 42 (Table 7).

Table 8 showed the effects of dietary RECC on the jejunum immune indexes in broilers. On d 21, the contents of IgM, IL-2 and IL-4 in jejunum showed a significant quadratic increasing effect with the increase of RECC dose (p < .05); IgG and IgM showed significant quadratic increase effect (p < .05), and secretory IgA (sIgA) had a significant linear or quadratic increase effect with the increase of RECC dose (p < .05); IL-2 and IL-4 showed a significant linear decrease (p < .01) or quadratic (p < .01, p < .01) increasing effect on d 42. Compared with the control group, the supplementation of RECC in broilers diet increased the secretion of immunoglobulin and anti-inflammatory factors, and the immune effect was the best when the supplemental level was 150 mg/kg and 200 mg/kg in the whole trial period.

**Immune related gene expression levels**

The mRNA expression levels of jejunum immune related factors were shown in Table 9. On d 21 of the experiment, TLR4 showed a significant linear (p < .05) or extremely significant quadratic (p < .01) decreasing effect with increasing dose of RECC. Meanwhile, MyD88 was linearly or quadratically decreased (p < .01), also NF-κB p65 and IL-6 were linearly or quadratically reduced (p < .05), and NF-κB p50 showed a significant linear reduction effect (p < .01). On d 42, the mRNA expression levels of TLR4, NF-κB p65, NF-κB p50 and IL-6 decreased significantly with the increase of RECC dose (p < .01), and MyD88 showed a
Compared with CON group, adding RECC could inhibit TLR4/NF-κB pathway at the later stage of the experiment, and the inhibiting effect was the best at 200 mg/kg. This may be related to changes in intestinal structure and microbial community caused by rare earth elements and chitosan. Relevant studies have found that chitosan, as an animal cellulose, can significantly increase the height of intestinal villi, reduce crypt depth, promote the growth of beneficial bacteria in intestinal tract, inhibit reproduction of harmful bacteria, thus improving intestinal function and promoting growth in animals (Zhang et al. 2019, 2020). Lan et al. (2020) found that chitosan oligosaccharides could increase the relative number of beneficial bacteria in the intestinal tract of broilers, enhance their biological barrier, nutrient absorption and immune capacity, and inhibit the colonisation of harmful bacteria, thus improving the growth performance of broilers. In addition, rare earth elements are generally tasteless, so the palatability will not be affected, and feed intake hasn’t increased. Therefore, dietary RECC supplementation may promote animal growth through its effect on intestinal microecology. This provides a theoretical basis for exploring the relationship between RECC and intestinal microbes in the future.

Reactive oxygen species (ROS), including superoxide ions ($O_2^-$), hydrogen peroxide ($H_2O_2$) and hydroxyl radicals (OH), are metabolites of the body under normal conditions. They can be used as signal molecules to regulate a variety of biochemical pathways and physiological processes. However, when stimulated by external adverse factors, excessive ROS will lead to oxidative stress and irreversible damage to the body. As an important part of the antioxidant system in organisms, enzymatic and non-enzymatic systems can decompose too much ROS to maintain the balance of the antioxidant system. Among them, enzymatic antioxidants include SOD, GSH-Px and CAT. SOD is mainly located in the cytoplasm and mitochondrial matrix, and can rapidly convert $O_2^-$ into $H_2O_2$. Meanwhile, GPx and CAT in cells can decompose $H_2O_2$ into $H_2O$ to prevent the aggregation of $H_2O_2$. However, when the level of $H_2O_2$ increases uncontrollably, OH$^-$ will react with metal ions (Fe$^{2+}$) and irreversibly oxidise lipid, protein, DNA and other biological macromolecules, which will result in tissue damage and affect the health and growth of organisms. The levels of MDA in different biological fluids reflect the antioxidant capability and the degree of cellular damage caused by ROS (Bhagat et al. 2016; Richter and Kietzmann 2016; Winterbourn et al. 2016; Glorieux and Calderon 2017; Cui et al. 2018; Ogłodek 2018; Ghonimi et al. 2021). In this experiment, dietary RECC supplementation increased T-AOC in serum (d 42), T-AOC (d 21) and the activity of CAT, SOD and GSH-Px (d 42) in

### Table 9. Effects of RECC on the gene expression of jejunum immune related factors in broilers.

| Dietary RECC level (mg/kg) | p-value |
|---------------------------|---------|
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liver of broilers, and the appropriate dose was 200–250 mg/kg. In addition, MDA content in the RECC supplemental group decreased linearly or quadratically during the trial period. These results suggested that adding RECC could improve the antioxidant capacity of broilers. Up to now, there have been no studies about the effect of RECC on antioxidant capacity in poultry. However, studies on the effect of rare earth elements and chitosan on antioxidant capacity of animals and the underlying mechanism have been relatively mature. Previous studies showed that lanthanide elements could enhance the activity of CAT and SOD, which could decrease ROS content in cytoplasm, alleviate protein and DNA damage induced by oxidative stress (Bryant et al. 2016; Allawadhi et al. 2020; Li et al. 2020). As a natural polysaccharide, chitosan is a crucial macromolecule which affects the antioxidant system (Muthu et al. 2021). Numerous studies showed that chitosan could improve the activity of enzymes and non-enzymes in the antioxidant system of animals, and reduce the level of lipid peroxidation caused by ROS (Toz and Değer 2017; Bhoopathy et al. 2020; Wei et al. 2020). In addition, Mei et al. (2021) found that the regulation of chito-oligosaccharides on oxidative stress was achieved by improving intestinal barrier function. In view of the above, we speculate that lanthanide and chitosan, as active substances in RECC, can enhance the activity of enzymes and non-enzymes in the antioxidant system, reduce the level of lipid peroxidation and regulate the gastrointestinal barrier function to relieve oxidative stress. From the molecular level of view, the expression levels of antioxidant-related genes determine the antioxidant capacity of the organism. Nrf2 is a key transcription factor in the regulation of oxidative stress response and can induce the gene expression of downstream factors HO-1, SOD, CAT and GPx-7, thus alleviate the excessive production of ROS. When the body is stimulated, Nrf2 and Keap1 are rapidly decomposed and transferred to the nucleus, where they combine with the antioxidant reaction elements in the nucleus to initiate gene expression of related antioxidant proteins (such as SOD, GSH-7, HO-1, et al.), thus improving the antioxidant defense system of the cells (Song et al. 2018; Ren et al. 2019; Xie et al. 2019). In this experiment, the expression of Nrf2 increased with the increase of RECC addition, which activated the gene expression of related antioxidant enzymes. Consistent with the activity of antioxidant enzymes measured previously, the gene expression of CAT, SOD and GPx-7 increased quadratically, but the gene expression of antioxidant enzymes began to decrease when the dosage of RECC exceeded 200 mg/kg. It was found that the addition of other derivatives of chitosan in the diet also increased the activity and the gene expression level of antioxidant enzymes (Tao et al. 2019). Combined with the above results, we believe that the Nrf2 pathway is activated by RECC because chito-oligosaccharide generated after enzymatic hydrolysis of chitosan in the intestine can participate in the body reaction through intestinal mucosa. In addition, some studies have shown that chitosan can activate Nrf2 pathway through MAPK pathway (Zhang et al. 2019). Therefore, the specific regulation mechanism of RECC on animal antioxidant needs to be confirmed by further studies in the future.

As the main immunoglobulins in the organism, IgG, IgM and slgA can resist the intrusion of a variety of pathogens and toxins. IgG plays a role in humoral immunity, which has the ability to identify countless antigens and combine with them, and can activate the innate immune mechanism. IgM is produced by B cells, with high affinity, high agglomeration and effective complement activation, and can be converted to IgG, IgA in a particular case. As the primary mucosal antibody, slgA is secreted into the intestinal cavity constantly, with the function of removing pathogens, inhibiting virus growth and protecting the intestinal barrier. In addition, slgA can also combine the intestinal microbial community to form a microorganism colonisation to ensure the diversity of communities (Heyman and Shulman 2016; Thomson 2016; Sousa-Pereira and Woof 2019; Huus et al. 2021). In the present study, RECC supplementation increased IgA (d 21) and IgG (d 42) levels in serum. The contents of slgA (d 42), IgG (d 42) and IgM in jejunum also increased, suggesting that RECC could activate humoral immunity and improve immune function of broilers. Similar to this test results, the addition of chitosan could increase the content of IgA, IgG and IgM in the serum of Huoyan geese, and activate the secretion of complement, thereby improving immunity (Miao et al. 2020). However, some studies showed that the addition of β-glucan could reduce the level of slgA over-produced in intestinal, which might be attributed to improved intestinal barrier function and immune capacity (Wang et al. 2016). It is worth mentioning that IgA can activate immune function by regulating the production of TNF, IL-1β and other key cytokines by human myeloid immune cells, which is a key step in the control of local and systemic immunity (Hansen et al. 2019). Therefore, the cytokine content in serum and jejunum was measured in this test. The results showed that serum IL-1β and IL-6 decreased linearly.
Conclusions

In summary, dietary RECC improved immune and anti-
oxidant capacity and promoted growth of broilers. Under
the conditions of this experiment, the optimal
supplemental level of RECC in diet was
175–200 mg/kg.

Ethic approval

The care and use of laboratory animals reported in
this study were approved by the Inner Mongolia
Agricultural University’s Animal Care and Use
Committee and the Ministry of Agriculture of China
(GB/T 35892-2018).

Disclosure statement

No potential conflict of interest was reported by
the author(s).

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