The Systemic Zinc Homeostasis Was Modulated in Broilers Challenged by *Salmonella*

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**Abstract**

*Salmonella* challenge leads to systemic responses that induce the hypozincaemia in mice, which is considered a vital strategy against *Salmonella* invasion. However, it is not yet known if this phenomenon occurs in broilers. To investigate the change in zinc homeostasis of broilers against *Salmonella* challenge, 1-day-old male broilers were fed with the basal diet for 7 days. Afterwards, broilers were orally inoculated with either 0 or 0.5 × 10^8 CFU *Salmonella Typhimurium* (ST). The serum and selected tissues of *Salmonella*-challenged and non-challenged broilers were collected at 1, 3 and 7 days post-challenge for zinc homeostasis analysis. Our results showed that *Salmonella* challenge results in hypozincaemia (serum zinc decrease and liver zinc increase) via modulating the systemic zinc homeostasis of broilers. A profound, zinc transporter–mediated zinc absorption and redistribution affecting zinc homeostasis provided a mechanistic explanation for this phenomenon. In addition, we found that the zinc importers Zip5, Zip10, Zip11, Zip12, Zip13 and Zip14 were mainly downregulated in *Salmonella*-challenged broilers to reduce zinc absorption in the duodenum, while the Zip14 mRNA expression was upregulated to redistribute zinc into the liver. Collectively, these findings reveal that broilers counteract *Salmonella* infection via modulating their systemic zinc homeostasis.

**Keywords** *Salmonella* · Broiler · Zinc · Hypozincaemia · Zinc homeostasis

**Introduction**

Food-borne *Salmonella* remains a major public health concern worldwide, being responsible for hundreds of millions of cases of human gastroenteritis [1–3]. Broiler meat contaminated with *Salmonella* is the primary vehicles for human salmonellosis [4, 5]. Aside from its impact on human health, *Salmonella* infection results in growth depression, intestinal inflammation, high mortality and cross-contamination in broilers [1, 6], which causes substantial economic loss to the poultry industry per year.

In mice, *Salmonella* infection induces hypoferaemia (serum iron decrease and liver iron increase), as iron plays a role in the regulation of the inflammatory response [7, 8]. Since other critical physiological functions also involve iron, all living organisms require iron to survive, including *Salmonella* [9]. The functions of hypoferaemia are considered to be the host defensive, because it decreases the availability of iron for *Salmonella* in a process termed “nutritional immunity” [10–13]. Besides iron, zinc also plays vital roles in host nutritional immunity [14, 15]. Similarly, hypozincaemia has also been observed after acute administration of numerous pathogens and agents, such as *Mycobacterium tuberculosis*, IL-6 and LPS [16–18]. This process is accompanied by a decrease in the serum zinc concentration and an increase in the zinc content in the liver due to the altered activity of zinc transporters, especially upregulation of Zip14 gene expression [16]. Meanwhile, there is an increased expression of zinc-binding protein metallothionein (MT) via a...
mechanism associated with oxidative stress [16, 19]. Notably, enhancing MT expression availability controls the “free zinc” (labile zinc that is available for binding by newly synthesized zinc metalloproteins) concentration in cells, and limits Salmonella infection in macrophages [20]. Thus, hypozincemia has been considered an effective strategy to limit pathogens from acquiring sufficient zinc for infection and proliferation in mice [16]. Interestingly, a similar phenotype was also observed in broilers under Escherichia coli or LPS stimulation [21]. In this context, broilers could also use hypozincemia as a useful defense strategy against pathogen infection. However, this has not yet been studied in broilers under Salmonella infection, and the roles and mechanisms of hypozincemia in broilers are also largely unknown.

Therefore, in this study, we investigated the impact of Salmonella challenge on the systemic zinc homeostasis of broilers and revealed how broilers modulate their zinc homeostasis to counteract Salmonella infection.

Materials and Methods

Animals and Diets

A total of 48, 1-day-old Arbor Acres (AA) male broilers were fed the basal diet for 7 days. Afterwards, the broilers were randomly divided into two treatment groups: non-challenged control group; Salmonella-challenged group. The basal diet (Table 1) was formulated to meet the requirements recommended by the National Research Council. All broilers were placed in a single thermo-controlled room. Room temperature was maintained at 32 °C during the first 3 days of life and then decreased by 2 to 3 °C per week. Broilers were given ad libitum access to feed and water and 24-h illumination throughout the whole experimental trial. The experimental procedures used in this study were approved by the Animal Care Advisory Committee of Sichuan Agricultural University.

Oral Salmonella Inoculation

On day 7, broilers were orally inoculated with either 0 or $0.5 \times 10^8$ CFU Salmonella enterica serovar Typhimurium (ST), according to the previous assignment (non-challenged vs. challenged). The method is detailed elsewhere [22]. The strain of ST used in this experiment was from the American Type Culture Collection (ATCC, No. 14028).

Growth Performance

The body weight of broilers was recorded at 7, 8, 10 and 14 days of age. These values were used to calculate the average body weight gain, according to the body weight of each growth phase.

Sample Collection and Procedures

At 1, 3 and 7 days post-challenge (at 8, 10 and 14 days of age), blood samples were taken from eight randomly selected birds in each group, and centrifuged at 2500 g/min for 10 min at 4 °C and then serum layer stored at −20 °C for serum zinc concentration analysis. Afterwards, the broilers were sacrificed by CO2 to collect the liver, spleen, thymus, bursa of Fabricius, duodenum, jejunum, ileum and cecum for the determination of zinc content and expression levels of zinc metabolism-related genes. Note that selected birds had fasted 12 h before sample collection.

RT-PCR

Total RNA was extracted from the liver and duodenum using RNAiso Plus reagent (TaKaRa), according to the manufacturer’s protocol and transcribed into cDNA by using the Prime Script™ RT reagent kit (TaKaRa). Quantitative real-time PCR system was performed on a CFX96 PCR system (BioRad) with the oligonucleotide sequences shown in Table S1. Relative gene expression was calculated with the $2^{\Delta\Delta Ct}$ method [23], normalizing the results to the house-keeping gene $\beta$-actin.

Zinc Measured by Inductively Coupled Plasma Mass Spectroscopy

An Agilent 7500cx inductively coupled plasma mass spectroscopy (ICP-MS) instrument (G3148B ISIS, Agilent Technologies, Japan) equipped with a G3160B I-AS integrated autosampler was employed to measure the ion profile since it allows a reduction in the detection time and volume of each sample compared with similar instruments. The typical operating conditions and the pretreatments of samples used in this study have been described previously [24].

Statistical Analysis

Statistical analysis was performed using GraphPad Prism software (Version 5.01). All results were presented as mean ± SEM. Statistical tests included the unpaired two-tailed Student’s $t$ test as appropriate with Bonferroni post hoc tests. Significance ($P$ value) was evaluated at the 0.05 level.
Table 1 Composition and nutrient concentrations of the diet (air dry-basis, %)

| Ingredients                  | Amount | Calculated nutrient concentrations | Amount |
|-----------------------------|--------|------------------------------------|--------|
| Corn                        | 54.30  | Metabolisable energy (kcal/kg)     | 2950.00|
| Soybean meal                | 38.12  | Crude protein                      | 21.00  |
| Soybean oil                 | 3.40   | Calcium                            | 1.01   |
| L-Lysine hydrochloride      | 0.15   | Non-phytate phosphorus             | 0.45   |
| DL-Methionine               | 0.25   | Lysine                             | 1.15   |
| Calcium carbonate           | 1.14   | Methionine                         | 0.50   |
| Dicalcium phosphate         | 1.86   | Methionine and cystine             | 0.86   |
| Sodium chloride             | 0.40   |                                    |        |
| Choline chloride            | 0.15   |                                    |        |
| Premixa                     | 0.23   |                                    |        |

*Supplied the following per kilogram of complete feed: Cu (CuSO₄·5H₂O), 8 mg; Fe (FeSO₄·7H₂O), 100 mg; Mn (MnSO₄·7H₂O), 120 mg; Zn (ZnSO₄·7H₂O), 80 mg; Se (Na₂SeO₃), 0.3 mg; I (KI), 0.70 mg; vitamin A (retinyl palmitate), 8000 IU; cholecalciferol, 1000 IU; vitamin E (DL-tocopheryl acetate), 20 IU; thiamine, 0.8 mg; riboflavin, 2.5 mg; pyridoxine, 1.5 mg; pantothenic acid, 2.2 mg; folic acid, 0.55 mg; nicotinic acid, 35 mg; and biotin, 0.18 mg.

Fig. 1 The growth performance of 7~14 day-old broilers. a The average body weight of broilers at 7, 8, 10 and 14 days of age. b The average body weight gain of broilers at 1, 3 and 7 days post-challenge (n = 8). *P < 0.05, **P < 0.01, ***P < 0.001, all data compare with control, the same with the follow figures.
Results

Salmonella Challenge Decreased the Growth Performance of Broilers

Our results showed that there was no significant difference in the average body weight between the control and Salmonella-challenged broilers (Fig. 1a), with only a slight tendency towards a decreased body weight of broilers at 14 days ($P = 0.0953$). In contrast, Salmonella challenge dramatically reduced the average body weight gain of broilers at 3 and 7 days post-challenge (Fig. 1b).

Hypozincæmia Was Observed in Salmonella-Challenged Broilers

Generally, mice challenged with Salmonella display profound changes in their metal metabolism [25]. In the case of zinc, “hypozincæmia” is among the changes observed in the period of acute inflammatory response. It is considered an effective strategy for mice to combat Salmonella challenge [16]. In broilers, hypozincæmia was also observed following Salmonella challenge (Fig. 2). Salmonella challenge resulted in a serum zinc decrease at 3 days post-challenge (Fig. 2a) and a liver zinc content increase, zinc was redistributed into the liver at 1 day post-challenge (Fig. 2b).

Zinc Was Also Redistributed into the Bursa of Fabricius

As noted above, zinc was redistributed into the liver in Salmonella-challenged broilers (Fig. 2), which is considered to be a response by the host defence system. A great deal of literature has already revealed that immune organs play crucial roles in the defence against Salmonella [22]. Whether the host will alter the zinc metabolism of their immune organs in response to Salmonella challenge remains unknown until now. We checked the zinc content in three different immune organs of broilers. As shown in Fig. 3, Salmonella challenge altered the zinc metabolism in the spleen and bursa of Fabricius. However, there was no difference in the content of zinc in the thymus (Fig. 3b). Intriguingly, Salmonella challenge slightly reduced the zinc content in the spleen (Fig. 3a), but significantly increased the zinc content in the bursa of Fabricius, suggesting that zinc was also redistributed into the bursa of Fabricius in Salmonella-challenged broilers.

Salmonella Challenge Inhibited the Zinc Absorption in the Intestine

As zinc is not stored in body, it must to be ingested daily and its homeostasis must to be accurately regulated. Whether the host mediates the hypozincæmia against Salmonella challenge through limiting the absorption of zinc was not fully known. Therefore, the duodenal, jejunal, ileal and cecal contents of zinc were measured by ICP-MS in this study. As shown in Fig. 4,
Salmonella challenge inhibited zinc absorption in the duodenum, and the zinc content of the duodenum in Salmonella-challenged broilers was less compared with the control group at the 1 day post-challenge (Fig. 4a). Similar results were also observed in the ileum (Fig. 4c). Interestingly, Salmonella challenge resulted in zinc accumulation in the jejunum and cecum at 3 days post-challenge (Fig. 4b, d).

**Zinc Transporter-Mediated Hypozincaemia in Salmonella-Challenged Broilers**

Figures 2b and 4a showed that Salmonella challenge altered the zinc homeostasis of the liver and duodenum, which plays a crucial role in regulating the systemic zinc homeostasis. The MT mRNA expression was significantly upregulated in the liver at 1 day post-challenge (Fig. 5a). In addition, the host upregulated Zip14 (a zinc importer) mRNA expression to accumulate zinc in the liver (Fig. 5a). On the contrary, the mRNA expressions of zinc exporters ZnT1, ZnT4, ZnT5, ZnT6, ZnT8 and ZnT9 in the liver were significantly downregulated in Salmonella-challenged broilers. Meanwhile, Salmonella challenge caused a significantly decrease in MT mTNA expression that was accompanied by differential expression of specific zinc transporters in the duodenum at 1 day post-challenge (Fig. 5b). The host limited the zinc absorption in the duodenum by downregulating the mRNA expression of zinc importers, such as Zip5, Zip9, Zip10, Zip11, Zip12, Zip13 and ZIP14, and decreasing the mRNA expression levels of the zinc exporters ZnT1, ZnT4, ZnT6 and ZnT7 mRNA expression (Fig. 5b).

**Discussion**

Salmonella challenge results in diarrhoea and severely reduces the body weight of animals [1, 26]. The impact of Salmonella challenge on the performance of broilers has been reported and
was further confirmed in this study. *Salmonella* challenge substantially decreased the body weight gain of *Salmonella*-challenged broilers at 3 and 7 days post-challenge. *Salmonella* challenge impairs the intestinal mucosal barrier and affects the absorption, transfer and utilization of nutrients of the host, which explains this phenomenon [27, 28].

One of the most characteristic features of the acute-phase response to pathogen challenge is a dramatic change in the metabolism of ions, mainly transition metal ions, such as iron, zinc, copper and manganese [25], which are essential for host and pathogen. Consequently, the host has evolved sophisticated sequestration mechanisms to limit pathogen access to these ions [10]. These processes of host-enforced micronutrient restriction are termed “nutritional immunity” [10, 12]. Hypozincæmia induced by *Salmonella* challenge is believed to belong to the defence arsenal of nutritional immunity [16]. In line with the literature evidence, we revealed that *Salmonella*-challenged broilers also display hypozincæmia.

**Fig. 4** *Salmonella* challenge inhibited the zinc absorption in the small intestine. Duodenal (a), jejunal (b), ileal (c) and cecal (d) zinc content of broilers at 1, 3 and 7 days post-challenge were detected by ICP-MS (*n* = 8)
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Zinc was redistributed into the liver during the process of acute-phase response to Salmonella challenge, which was also confirmed by the MT mRNA expression in the liver. In addition, we found that the host upregulated zinc importer Zip14 gene expression to redistribute zinc into the liver and downregulated the gene expression levels of zinc exporters (ZnT4, ZnT5, ZnT6, ZnT8 and ZnT9) to accumulate zinc into the liver. Hypozincaemia is beneficial to reduce zinc availability for Salmonella, which limits Salmonella replication and formation of virulence gene formation [29, 30] and redistributes zinc into the liver for hepatic synthesis of acute-phase response proteins [16, 31]. Thus, it is not surprising that hypozincaemia is an important innate defence strategy.

Accumulating literature evidence shows that immune organs play crucial roles in defence against Salmonella infection [32–34], and mild zinc alteration dramatically affects the function of immune organs [35]. Therefore, we measured the zinc content in the spleen, thymus and bursa of Fabricius. As expected, Salmonella infection causes zinc redistribution into the bursa of Fabricius, a primary central humoral immune organ responsible for establishment and maintenance of the B cell compartment in avian species [36, 37]. Zinc accumulation contributes to B cell proliferation and enhances the immune function of the host to against bacterial infection. Furthermore, we also found substantial changes among other ions in the bursa of Fabricius of Salmonella-challenged broilers (data not shown). However, no remarkable changes in zinc were observed in the spleen and thymus.

As zinc is not stored in the body, it has to be ingested daily and its homeostasis needs to be regulated accurately. Salmonella challenge inhibited zinc absorption in the duodenum via downregulation of zinc importer (Zip5, Zip10, Zip11, Zip12, Zip13 and Zip14) mRNA expression, which locate at membranes of cells in mammals and are responsible for zinc absorption from the gut tract. Notably, zinc exporters ZnT1, ZnT4, ZnT6 and ZnT7 were also downregulated in the duodenum of Salmonella-challenged broilers. These zinc exporters locate at the basement membrane or organelles in the duodenum and contribute to transport zinc from the intestinal epithelium to blood [38–41]. Hence, downregulation of these zinc exporters will significantly lessen the serum zinc concentration, which provides a mechanistic explanation for hypozincaemia.

Overall, we found that the systemic zinc homeostasis of broilers was modulated by Salmonella. Salmonella challenge induced hypozincaemia via limiting zinc absorption in the duodenum and redistributing zinc into the liver and bursa of Fabricius. Zinc transporters play a crucial role in this process, especially ZIP14. These changes in broilers seem to belong to the defence arsenal of the host.

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