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Manganese Gluconate as a Safe Trace Element Supplement Enhances Immune Function and Resistance to Staphylococcus aureus Infection in Broilers

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Abstract

Manganese is an essential trace element with known immunomodulatory properties, yet the effects of organic manganese supplementation on disease resistance in poultry remain poorly understood. This study evaluated the safety and immunoprotective efficacy of orally administered manganese gluconate in yellow-feathered broilers. A total of 120 seven-day-old broilers were randomly allocated to four groups and orally administered either vehicle control or manganese gluconate at 1, 5, or 10 mg/kg body weight for 14 consecutive days. No adverse health responses were observed across all treatment groups, confirming the safety of manganese gluconate within this dosage range. At the optimal dosage, manganese gluconate significantly promoted splenic lymphocyte proliferation (CCK-8 assay), upregulated the expression of key cytokines (IL-2, IL-4, IL-10, TNF-α), improved serum antioxidant status (T-AOC, T-SOD, GSH-PX, MDA), enhanced specific anti-*S. aureus* IgG production, and strengthened peritoneal macrophage-mediated phagocytosis of *S. aureus*. Following intraperitoneal challenge with *S. aureus* Newman strain (1×10^{10} CFU/bird), manganese gluconate effectively reduced bacterial colonization in the liver, spleen, and kidney, and improved the survival rate of infected broilers. These findings demonstrate that manganese gluconate is a safe and effective nutritional supplement that enhances immune function and increases resistance to bacterial infection in broiler production.

1. Introduction

Manganese is an essential trace element that plays a critical role in maintaining physiological metabolism, immune function, and antioxidant defense in anima[1, 2]. As a key cofactor for manganese superoxide dismutase (MnSOD), manganese participates in the proliferation, differentiation, and functional regulation of immune cells[3, 4]. Inorganic manganese sources, such as manganese chloride and manganese sulfate, have been widely used in animal feed supplementation, but organic manganese sources, represented by manganese gluconate, have attracted increasing attention due to their higher bioavailability, better absorption efficiency, and milder physiological compatibility[5, 6]. However, despite these advantages, the effects of organic manganese supplementation on disease resistance in poultry remain poorly understood.

Staphylococcus aureus (*S. aureus*) is a major opportunistic pathogen in broiler production, causing severe clinical symptoms including omphalitis, acute septicemia, arthritis, and osteomyelitis, leading to substantial economic losses and animal welfare concerns[7, 8]. The widespread emergence of multidrug-resistant strains has created an urgent demand for safe, effective, and sustainable nutritional alternatives to antibiotics in poultry farming[9, 10].

Previous studies have shown that manganese supplementation enhances immune function and antioxidant capacity in various animal models[5, 11]. However, the application potential of manganese gluconate as a nutritional additive to enhance resistance against *S. aureus* infection in broilers remains largely unexplored. Critically, the safe supplementation dosage—a fundamental prerequisite for practical application—has not been systematically established[12, 13]. Furthermore, while the regulatory roles of

manganese in immune response and redox homeostasis are well documented in mammals, the mechanisms by which manganese gluconate modulates broiler immune function, antioxidant status, and further restricts *S. aureus* colonization and systemic spread remain unclear.

Therefore, this study was designed to comprehensively evaluate the application value of manganese gluconate in broiler production. The primary aim was to determine the safe supplementation dose of manganese gluconate for broilers. On this basis, we further evaluated its effects on immune regulation, antioxidant capacity, and protective efficacy against *S. aureus* challenge. The overall objective was to elucidate the regulatory effects of manganese gluconate on broiler health and pathogen resistance, providing a theoretical basis and practical reference for the rational application of organic manganese additives as promising antibiotic alternatives in intensive broiler production.

2. Materials and methods

2.1. Ethics Statement

All animal procedures were approved by the Institutional Animal Care and Use Committee of Heilongjiang Bayi Agricultural University (Approval No. SMKXJSXY2025029) and were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of Heilongjiang Bayi Agricultural University.

2.2. Bacterial strains and reagents

S. aureus strain Newman was stored in Heilongjiang Provincial Key Laboratory of Animal Cell Activities and Stress Adaptations, Heilongjiang Bayi Agricultural University. manganese gluconate was ordered from Macklin Biological Reagent Co., Ltd. (Shanghai, China) .Cell counting kit-8 (CCK-8) was purchased from Ranjeck Technology Co., Ltd. (Shanghai, China). RNA extraction kit, Reverse Transcriptase Kit and quantitative reverse transcription polymerase chain reaction (qRT-PCR) kit were ordered from Nanjing Vazyme Biotech Co., Ltd. (Nanjing, China). Total Antioxidant Capacity Assay Kit (T-AOC) was obtained from Beyotime Biotechnology Co., Ltd (Shanghai,China).Total Superoxide Dismutase Assay Kit(T-SOD), Lipid Peroxidation MDA Assay Kit (MDA) and Cellular Glutathione Peroxidase Assay Kit (GSH-PX) were ordered from Wanlei Life Sciences Co., Ltd (Shenyang, China).

2.3. Experimental design and animal treatment

A total of 120 one-day-old female yellow-feathered broilers were obtained from a commercial hatchery (Xingwang Commercial Hatchery, Heilongjiang, China). The basal diet (without antibiotic or manganese supplementation) was formulated by the Immunological Preparations and Response Regulation Laboratory of Heilongjiang Bayi Agricultural University to meet the nutritional requirements for broilers recommended by the National Research Council (NRC). The natural manganese content of the basal diet was 30.65 mg/kg (dry matter basis), as determined based on feed ingredient composition according to the Chinese Feed Composition and Nutritional Value Table (2024 edition).

At 7 days of age, broilers were randomly allocated into four groups (n = 30 per group). The control group received an oral gavage of vehicle (glucose solution), while the three treatment groups received manganese gluconate at doses of 1, 5, or 10 mg/kg body weight. All solutions were administered by oral gavage at a fixed volume of 500 μL per bird once daily for 14 consecutive days. Body weight was recorded weekly to monitor growth and adjust gavage volumes.

2.4. Preparation of inactivated bacteria

S. aureus Newman strain was cultured overnight in 50 mL of Tryptic Soy Broth (TSB) at 37°C in a shaking incubator. Bacterial cells were harvested by centrifugation at 5,000 rpm for 10 min at 4°C, washed three times with sterile phosphate-buffered saline (PBS), and resuspended in PBS. The bacterial concentration was adjusted to 1×10^9 CFU/mL based on optical density (OD_{600}) and confirmed by serial dilution and plate counting.

For inactivation, formaldehyde solution was added to the bacterial suspension to a final concentration of 0.5% (v/v), followed by incubation in a shaking incubator at 180 rpm and 37°C for 6 h. To confirm complete inactivation, 100 μL of the treated suspension was spread onto Tryptic Soy Agar (TSA) plates and incubated at 37°C for 24 h. No colony growth was observed, confirming the loss of bacterial viability. The inactivated bacterial suspension was then aliquoted and stored at -20°C for subsequent use in lymphocyte proliferation assays and antibody detection.

2.5. Safety assessment

During the 14-day oral administration period, broilers were monitored daily for clinical signs, including mental status, behavior, feed intake, feather condition, fecal consistency, respiration, and any signs of distress or illness. Body weight was recorded weekly. No adverse clinical reactions, abnormal behavioral performance, or mortality were observed in any of the manganese-supplemented groups. Based on these findings, the highest dose of 10 mg/kg BW was considered safe for manganese gluconate supplementation in broilers under the present experimental conditions.

2.6. Spleen lymphocyte proliferation

After the 14-day oral administration period, spleens were aseptically collected from three broilers randomly selected from each group. Single-cell suspensions were prepared by homogenizing the spleens and passing the homogenate through a 200-mesh cell strainer. The cell suspension was centrifuged at 1,000 rpm for 3 min, and the pellet was resuspended in 1 mL of red blood cell lysis buffer. After incubation for 2 min at room temperature, 9 mL of Dulbecco's Modified Eagle Medium (DMEM) was added to terminate lysis, followed by centrifugation at 1,000 rpm for 3 min. The cell pellet was washed three times with PBS.

Viable cells were counted using a hemocytometer, and the concentration was adjusted to 1×10^6 cells/mL. Then, 100 μL of the cell suspension (1×10^5 cells/well) was added to each well of a 96-well plate. Inactivated *S. aureus* Newman strain was added to a final concentration of 1×10^5 CFU/mL to stimulate lymphocyte proliferation. Cells were cultured in a 5% CO_2 incubator at 37°C for 48 h. After

incubation, cell proliferation was assessed using the Cell Counting Kit-8 (CCK-8) following the manufacturer's instructions. The absorbance was measured at 450 nm using a microplate reader. The stimulation index (SI) was calculated as the ratio of the OD value of stimulated wells to that of unstimulated control wells.

2.7. Quantitative real-time PCR (qRT-PCR)

After 14 days of continuous G-Mn gavage treatment, total RNA was extracted from broiler peripheral blood mononuclear cells (PBMCs) according to the manufacturer's instructions of the RNA extraction kit. Complementary DNA (cDNA) was synthesized using a reverse transcription kit. The mRNA expression levels of the target cytokines (IL-2, IL-4, IL-10, and TNF-α) were quantified by qRT-PCR, with GAPDH used as the internal reference gene. qRT-PCR reactions were performed using 2×ChamQ™ Universal SYBR qPCR Master Mix, and all primer sequences are listed in Table 4. Each sample was analyzed in triplicate. The relative mRNA expression levels were calculated using the $2^{\wedge -\triangle\triangle Ct}$ method, with the control group set as the baseline to evaluate alterations in target gene expression. All qRT-PCR assays were carried out on a fluorescence quantitative PCR system.

Table 1
Composition of the basal die. $CaHPO_4$, Calcium hydrogen phosphate, NaCl: Sodium chloride.

Ingredients	Cotent (%)
Corn	71.98
Soybean meal	23.00
Soybean oil	2.32
Limestone	1.2
CaHPO4	0.94
NaCl	0.15
Lysine	0.15
DL-Methionine	0.13
Threonine	0.13
Total	100

Table 3

Daily total manganese intake = manganese intake from basal diet + manganese gluconate (G-Mn) gavage intake, presented as "Mean ± standard deviation (Mean ± SD) (n = 10/group). Manganese intake from basal diet was calculated as: Daily feed intake (kg/bird) × natural manganese content of the basal diet (30.65 mg/kg). G-Mn gavage intake was calculated as: Daily body weight (kg/bird) × set gavage dose (Control group: 0 mg/kg BW; Group □: 1 mg/kg BW; Group □: 5 mg/kg BW; Group □: 10 mg/kg BW). The daily body weight was derived by linear growth model based on the recorded body weights at different stages (8, 14, 21, 28 days of age), ensuring that the gavage dose was dynamically adjusted with the broilers' body weight, which was consistent with the experimental design of "dosage based on body weight". BW: body weight; G-Mn: manganese gluconate.

Age Stage (days)	**Basal Diet Mn Intake (mg/bird/day)**	**Daily Total Mn Intake (mg/bird/day)**			
		Control	**1 mg/kg BW**	**5 mg/kg BW**	**10 mg/kg BW**
8–14	0.307 ± 0.031	0.307 ± 0.031	0.487 ± 0.034	1.187 ± 0.052	2.087 ± 0.076
15–21	0.858 ± 0.061	0.858 ± 0.061	1.170 ± 0.070	2.420 ± 0.105	3.970 ± 0.148
22–28	1.533 ± 0.092	1.533 ± 0.092	2.000 ± 0.108	3.862 ± 0.165	6.191 ± 0.234

Table 4

GAPDH: glyceraldehyde 3-phosphate dehydrogenase; IL-2: interleukin 2; IL-4: interleukin 4; IL-10: interleukin 10; TNF-α: Tumor Necrosis Factor-alpha

Target gene	**Gene ID**	**Primer sequence (5’-3’)**
GAPDH	NM_204305.2	F: CAGAACATCATCCCAGCGTCCAC
		R: CGGCAGGTCAGGTCAACAACAG
IL-2	NM_204153.2	F: CTCAAGAGTCTTACGGGTCTAAATCAC
		R: TCTCACAAAGTTGGTCAGTTCATGG
IL-4	NM_001007079.2	F: AGCCAGCACTGCCACAAGAAC
		R: CGTGGGACATGGTGCCTTGAG
IL-10	NM_001004414.4	F: AGCAAAGCCATCAAGCAGATCAAG
		R: CACTTCCTCCTCTCATCAGCAG
TNF-α	NM_204267.2	F: GACCGCCCAGTTCAGATGAGTTG
		R: TGTTTGATGGGCACGGAGTTGAC

2.8. Antioxidant evaluation

After the 14-day oral administration period, serum was separated from peripheral blood samples by centrifugation at 3,000 rpm for 10 min at 4°C. The levels of total antioxidant capacity (T-AOC), total superoxide dismutase (T-SOD), and glutathione peroxidase (GSH-PX), as well as the lipid peroxidation

product malondialdehyde (MDA), were measured using commercially available kits according to the manufacturer's instructions.

2.9. Antibody detection by indirect ELISA

Serum samples were collected from three randomly selected broilers per group after the 14-day oral administration period. Blood was obtained from the wing vein, and serum was separated by centrifugation. Indirect ELISA was performed to detect specific IgG antibodies against *S. aureus*. Inactivated *S. aureus* Newman strain was diluted to 1×10^8 CFU/mL in PBS, and 100 µL of the suspension was added to each well of a 96-well plate. The plate was incubated overnight at 4°C to coat the wells. After incubation, the wells were washed three times with PBST (PBS containing 0.05% Tween-20) and blocked with 100 µL of 5% skim milk at 37°C for 2 h, followed by three additional washes with PBST. Serum samples were diluted 1:1000 in PBS, and 100 µL of diluted serum was added to each well. After incubation at 37°C for 2 h, the wells were washed three times with PBST. Then, 100 µL of HRP-conjugated goat anti-chicken IgG (diluted 1:5000) was added to each well and incubated at 37°C for 1 h, followed by three washes with PBST. For color development, 100 µL of TMB substrate solution was added to each well and incubated in the dark at 37°C for 15 min. The reaction was stopped by adding 100 µL of 2 N H_2SO_4. The optical density (OD) was measured at 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

2.10. Extraction of peritoneal macrophages

Peritoneal macrophages were harvested from naive (non-treated) yellow-feathered broilers. To recruit macrophages, donor broilers were intraperitoneally injected with 5 mL of sterile sodium mercaptoacetate solution (60 mg/mL). After 72 h, peritoneal macrophages were collected under sterile conditions. Briefly, 10 mL of ice-cold sterile PBS was injected into the peritoneal cavity, and the abdominal wall was gently massaged for 2 min. The peritoneal fluid containing macrophages was aspirated and centrifuged at 1,000 rpm for 10 min at 4°C. The supernatant was discarded, and red blood cell lysis buffer was added to the cell pellet. After incubation at room temperature for 5 min, the mixture was centrifuged again (1,000 rpm, 10 min, 4°C). The resulting cell pellet was resuspended in complete RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cell concentration was adjusted to 5×10^5 cells/mL, and 1 mL of the cell suspension was added to each well of a 12-well plate. The plate was incubated at 37°C in 5% CO_2 for 2 h to allow macrophage adherence. Non-adherent cells were gently removed by washing twice with pre-warmed PBS, and the adherent peritoneal macrophages were used for the opsonophagocytosis assay.

2.11. Opsonophagocytosis assay

The ability of serum antibodies to mediate opsonophagocytosis of live *S. aureus* by peritoneal macrophages was evaluated. Serum samples were collected from manganese-treated broilers after the 14-day oral administration period (as described for antibody detection). Peritoneal macrophages were harvested from naive donor broilers by intraperitoneal injection of sodium mercaptoacetate (as described above). Live *S. aureus* Newman strain was cultured overnight in Tryptic Soy Broth (TSB) at

37°C. Bacterial cells were harvested by centrifugation at 5,000 rpm for 10 min, washed three times with sterile PBS, and resuspended in PBS. The bacterial concentration was adjusted to 2×10^6 CFU/mL based on optical density (OD_{600}) and confirmed by serial dilution and plate counting. Then, 2×10^6 CFU of live *S. aureus* was mixed with 30 μL of serum from each experimental group. The mixture was incubated at 37°C for 2 h to allow opsonization. The opsonized bacterial mixture was then added to the wells of a 12-well plate containing adherent peritoneal macrophages (5×10^5 cells/well) and co-incubated at 37°C for 2 h. After co-incubation, the wells were washed three times with sterile PBS to remove non-adherent bacteria, and 100 μg/mL gentamicin was added to each well to kill extracellular bacteria. Following incubation at 37°C for 1 h, the wells were washed three times with PBS to remove residual gentamicin. Macrophages were then lysed with sterile distilled water to release intracellular bacteria. The cell lysates were serially diluted, spread onto Tryptic Soy Agar (TSA) plates, and incubated at 37°C for 12 h. The number of viable *S. aureus* colonies was counted, and the opsonophagocytic activity was calculated based on the recovered CFU.

2.12. Bacterial load determination

After 14 days of continuous oral administration, broilers were intraperitoneally challenged with 1 mL of *S. aureus* Newman strain suspension (5×10^8 CFU/mL). At 48 h post-challenge, three broilers were randomly selected from each group. The liver, spleen, and kidney were aseptically collected, weighed, and homogenized in sterile PBS (1:10, w/v) to prepare tissue homogenates. The homogenates were serially diluted ten-fold with sterile PBS. An aliquot of 20 μL from each dilution was spread onto Tryptic Soy Agar (TSA) plates and incubated at 37°C overnight. The number of viable *S. aureus* colonies was counted, and the bacterial load was calculated as CFU per gram of tissue (CFU/g).

2.13. Broilers challenge and survival rate observation

After 14 days of continuous oral administration, 10 broilers from each group were intraperitoneally challenged with 1 mL of *S. aureus* Newman strain suspension (1×10^{10} CFU/mL). Broilers were monitored daily for 14 days post-challenge for clinical signs, mental status, and mortality. The survival rate for each group was calculated as: (number of surviving broilers / total number of challenged broilers) × 100%.

2.14. Data statistics and analysis

Data was analyzed with GraphPad Prism 9.5 data software. Differences between groups were determined using a two-tailed unpaired Student's t-test. Statistical significance was denoted as * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

3. Result

3.1. Safety profile of G-Mn in broilers within the tested dosages

During the 14-day oral administration period, no adverse clinical signs were observed in any of the manganese-supplemented groups. Broilers in all groups responded promptly to external stimuli, exhibited normal behavior, and showed no signs of illness. Feathers remained plump, clean, and lustrous. Feces were normal with no diarrhea, and respiration remained stable. Feed intake was consistent across all groups, with no significant differences in feeding speed or enthusiasm, and no instances of feed refusal. Body weight was recorded weekly, and no significant differences were observed among the four groups at any time point (Table 2). These results confirm that manganese gluconate is safe for broilers within the tested dosage range (1–10 mg/kg).

Table 2
Average weekly weight(g) (mean ± standard deviation). BW: body weight, BWG: body weight gain, Note: there were no significant differences among treatments ($p > 0.05$; ANOVA, followed by Tukey's post-hoc test). Control: Phosphate buffer solution (PBS); □: 1mg/kg BW Manganese Gluconate Group; □: 5mg/kg BW Manganese Gluconate Group; □: 10mg/kg BW Manganese Gluconate Group

Group	Control	1 mg/kg BW	5 mg/kg BW	10 mg/kg BW
Initial BW	45.3 ± 3.2	44.9 ± 2.8	45.1 ± 3.0	45.0 ± 2.9
7Days	122.1 ± 6.5	120.8 ± 5.9	123.5 ± 6.2	121.5 ± 6.0
14Days	235.6 ± 9.8	233.2 ± 8.7	234.8 ± 9.0	237.1 ± 9.2
21Days	389.2 ± 11.3	386.5 ± 10.9	391.0 ± 11.5	388.3 ± 11.1
28Days	542.5 ± 15.6	539.8 ± 14.2	545.2 ± 16.1	541.3 ± 15.0
BWG	497.2 ± 14.8	494.9 ± 13.5	500.1 ± 15.2	496.3 ± 14.2

3.2. G-Mn Promotes Splenic Lymphocyte Proliferation

The proliferation capacity of splenic lymphocytes was assessed using the CCK-8 assay. As shown in Fig. 1A, the stimulation index (SI) in all three G-Mn treatment groups was significantly higher than that in the control group ($P < 0.001$). The SI increased in a dose-dependent manner, with the 10 mg/kg group exhibiting the highest SI among all groups. These results indicate that G-Mn significantly enhances splenic lymphocyte proliferation in broilers, with the optimal effect observed at 10 mg/kg.

3.3. G-Mn upregulates cytokine mRNA levels in broilers

The mRNA expression levels of IL-2, IL-4, IL-10, and TNF-α in broiler PBMCs were measured by qRT-PCR. As shown in Fig. 1B–1E, G-Mn treatment upregulated all four cytokines in a dose-dependent manner. The most pronounced upregulation was observed for IL-2 and IL-10, with the 10 mg/kg group showing the highest expression levels ($P < 0.001$ for both). IL-4 and TNF-α were also significantly increased, particularly at the 5 and 10 mg/kg doses ($P < 0.05$ or $P < 0.01$). These results indicate that G-Mn enhances both Th1-type (IL-2, TNF-α) and Th2-type (IL-4, IL-10) immune responses in broilers.

3.4. G-Mn Improves Serum Antioxidant Capacity in Broilers

Serum antioxidant parameters were measured after the 14-day treatment period. As shown in Fig. 2A-2D, G-Mn supplementation enhanced serum antioxidant capacity in a dose-dependent manner. Specifically, the activities of T-AOC, GSH-PX, and T-SOD were significantly increased in G-Mn-treated groups compared with the control group, with the 5 and 10 mg/kg groups showing the most pronounced effects ($P < 0.01$ for T-AOC and GSH-PX; $P < 0.05$ for T-SOD). In contrast, serum MDA levels, a key marker of lipid peroxidation, decreased progressively with increasing G-Mn dose, with significant reductions observed at all doses ($P < 0.01$ for 1 mg/kg; $P < 0.001$ for 5 and 10 mg/kg).

3.5. G-Mn Promotes Anti-*S. aureus* IgG Production

Serum levels of specific IgG against *S. aureus* were measured by indirect ELISA using inactivated *S. aureus* Newman strain as the coating antigen. As shown in Fig. 3A, G-Mn supplementation increased serum IgG levels in a dose-dependent manner. The 1 mg/kg group showed significantly higher IgG levels than the control group ($P < 0.05$), and the 10 mg/kg group exhibited the highest IgG level among all groups ($P < 0.01$). These results indicate that G-Mn enhances the humoral immune response to *S. aureus* in a dose-dependent manner.

3.6. G-Mn Enhances Serum Opsonophagocytic Activity

Antibody-mediated opsonophagocytic activity of broiler serum against *S. aureus* was evaluated using peritoneal macrophages isolated from naive donor broilers. As shown in Fig. 3B, serum opsonophagocytic activity was lowest in the control group. In contrast, the 1 mg/kg G-Mn group showed significantly enhanced activity compared with the control group ($P < 0.05$), and this effect was further strengthened in the 5 mg/kg group ($P < 0.001$). Notably, the 10 mg/kg group exhibited the strongest activity among all groups ($P < 0.001$).

3.7. G-Mn Reduces Bacterial Load in Organs of *S. aureus*-Challenged Broilers

Bacterial loads of *S. aureus* in the liver, spleen, and kidney are shown in Fig. 4A-4C. Compared with the control group, all G-Mn treatment groups exhibited significantly reduced bacterial colonization in the three tested organs. The bacterial load was markedly decreased in the 1 mg/kg group and was further reduced in the 5 and 10 mg/kg groups. Among all groups, the 10 mg/kg group showed the lowest *S. aureus* burden in visceral tissues ($P < 0.001$).

3.8. G-Mn Increases Survival Rate of Broilers Challenged with *S. aureus*

Broilers were challenged with a lethal dose of *S. aureus* (1×10^{10} CFU/bird), and survival was monitored for 14 days. As shown in Fig. 4D, the survival rates of the G-Mn treatment groups were 30% (1 mg/kg), 30% (5 mg/kg), and 40% (10 mg/kg), whereas all broilers in the control group died within 6 days post-

challenge. G-Mn-supplemented broilers exhibited milder clinical symptoms compared with the control group, with the 10 mg/kg group showing the best recovery.

4. Discussion

In this study, we demonstrated that oral administration of manganese gluconate at 1–10 mg/kg body weight for 14 days is safe for yellow-feathered broilers and enhances immune function and resistance to Staphylococcus aureus infection. To our knowledge, this is the first study to systematically evaluate the immunoprotective efficacy of manganese gluconate against a specific bacterial pathogen in broilers[14].

Establishing safety is a prerequisite for any feed additive. Our results showed that 14-day oral administration of manganese gluconate at doses up to 10 mg/kg did not cause any adverse clinical signs or mortality, consistent with previous reports on organic manganese sources in poultry[15]. The absence of growth inhibition or abnormal body weight loss further supports the safe application of manganese gluconate within this dosage range. Based on these findings, the highest dose of 10 mg/kg was considered safe and was used as the optimal dose for subsequent immune evaluations.

Manganese is an essential trace element for immune cell function. In mammals, manganese deficiency impairs lymphocyte proliferation and cytokine production[13, 16]. Here, we found that manganese gluconate significantly promoted splenic lymphocyte proliferation in a dose-dependent manner, consistent with the immunostimulatory role of manganese. Moreover, manganese gluconate upregulated the expression of both Th1-type (IL-2, TNF-α) and Th2-type (IL-4, IL-10) cytokines[17, 18], indicating a balanced immune activation rather than polarization toward a single Th response[19, 20]. This balanced modulation may be particularly advantageous for host defense against *S. aureus*, which requires coordinated activation of both cellular and humoral immunity.[21]

Consistently, manganese gluconate significantly increased serum levels of specific anti-*S. aureus* IgG and enhanced antibody-mediated opsonophagocytic activity[22, 23]. The positive correlation between IgG levels and opsonophagocytic activity suggests that manganese gluconate promotes B cell differentiation and antibody production, which in turn facilitates macrophage-mediated bacterial clearance[24, 25]. These findings align with a previous study showing that manganese gluconate chloride enhances macrophage phagocytosis in broilers.

Manganese serves as a critical cofactor for manganese superoxide dismutase (MnSOD), a key mitochondrial antioxidant enzyme[26, 27]. In this study, manganese gluconate supplementation significantly increased serum T-AOC, T-SOD, and GSH-PX activities while decreasing MDA levels, indicating reduced oxidative stress. By improving the redox environment, manganese gluconate may create favorable conditions for immune cell activation and function[28, 29]. This dual effect—direct immunomodulation plus indirect antioxidant support—likely underlies the enhanced resistance to *S. aureus* infection observed in this study.

The most compelling finding of this study is the marked reduction in bacterial loads in the liver, spleen, and kidney, as well as the improved survival rate following lethal *S. aureus* challenge. The 10 mg/kg dose consistently showed the strongest protective effect across all measured parameters. The enhanced bacterial clearance is likely attributable to the improved immune and antioxidant status, as manganese gluconate-treated broilers exhibited higher lymphocyte proliferation, balanced cytokine expression, elevated specific IgG, enhanced opsonophagocytosis, and reduced oxidative stress before challenge.

Several limitations should be acknowledged. First, the molecular mechanisms by which manganese gluconate modulates immune cell function—such as its effects on NF-κB or MAPK signaling pathways—remain to be elucidated[30, 31]. Second, this study used a single broiler breed (yellow-feathered); further studies are needed to evaluate the efficacy of manganese gluconate in other commercial breeds. Third, field studies under commercial production conditions are warranted to confirm the practical applicability of manganese gluconate as an antibiotic alternative.

5. Conclusion

In conclusion, this study demonstrates that oral administration of manganese gluconate at 1–10 mg/kg body weight is safe for broilers. At the optimal dose of 10 mg/kg, manganese gluconate enhances immune function (lymphocyte proliferation, cytokine expression, specific IgG production, and opsonophagocytosis) and antioxidant capacity, leading to reduced bacterial loads and improved survival after *S. aureus* challenge. These findings support the potential application of manganese gluconate as a beneficial nutritional supplement to improve health status and control bacterial infection in broiler production.

Abbreviations

G- Mn
Manganese gluconate
S.aureus
Staphylococcus aureus
BW
Body weight
ELISA
Enzyme-linked immunosorbent assay
SI
Stimulation index
CFU
Colony-forming units
TMB
3, 3', 5, 5'-tetramethylbenzidine
CCK-8

Cell Counting Kit-8
TSA
Tryptic soy agar
TSB
Trypsin soy meat broth
PBS
Phosphate bufer solution

Declarations

CRediT authorship contribution statement

Jinzhu Ma: Writing – review, Investigation. **Changquan Liu**: Writing – original draft & Investigation. **Shuyu Wei**: Original draft, Investigation. **Ming Xu**: Resources, Investigation. **Mingyang Tao**: Data Curation. **Youxi Fu**: Methodology. **Kexin Liu**: Methodology. **Siqi Wang**: Project Administration. **Simiao Yu**: Validation. **Beiyan Wang**: Supervision, Formal analysis. **Liquan Yu**: Methodology. **Baifen Song**: Formal analysis.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Data availability

Data will be made available on request.

Ethical statement

This study was performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Heilongjiang Bayi Agricultural University. The experimental protocols were approved by the Institutional Animal Care and Use Committee at Heilongjiang Bayi Agricultural University.

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Figures

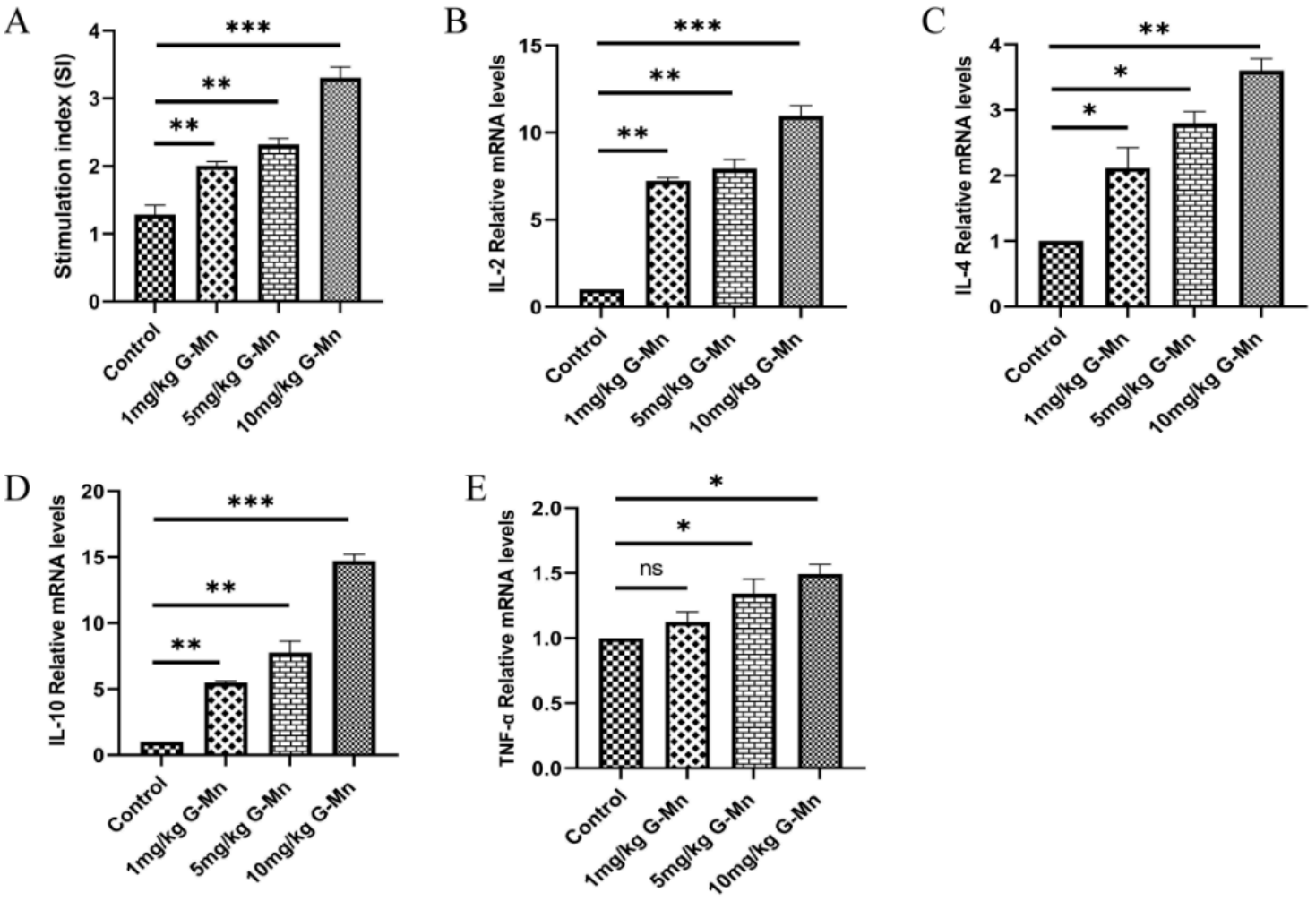


Figure 1

Effects of graded dietary G-Mn supplementation on splenic lymphocyte proliferation and cytokine mRNA expression in broilers. (A) Splenic lymphocyte proliferation index (SI). (B-E) Relative mRNA expression levels of IL-2 (B), IL-4 (C), IL-10 (D), and TNF-α (E) in peripheral blood mononuclear cells (PBMCs). Data are presented as the mean ± standard error (SE). Asterisks indicate significant differences compared with the control group (*P < 0.05, **P < 0.01, ***P < 0.001); ns, no significant difference.

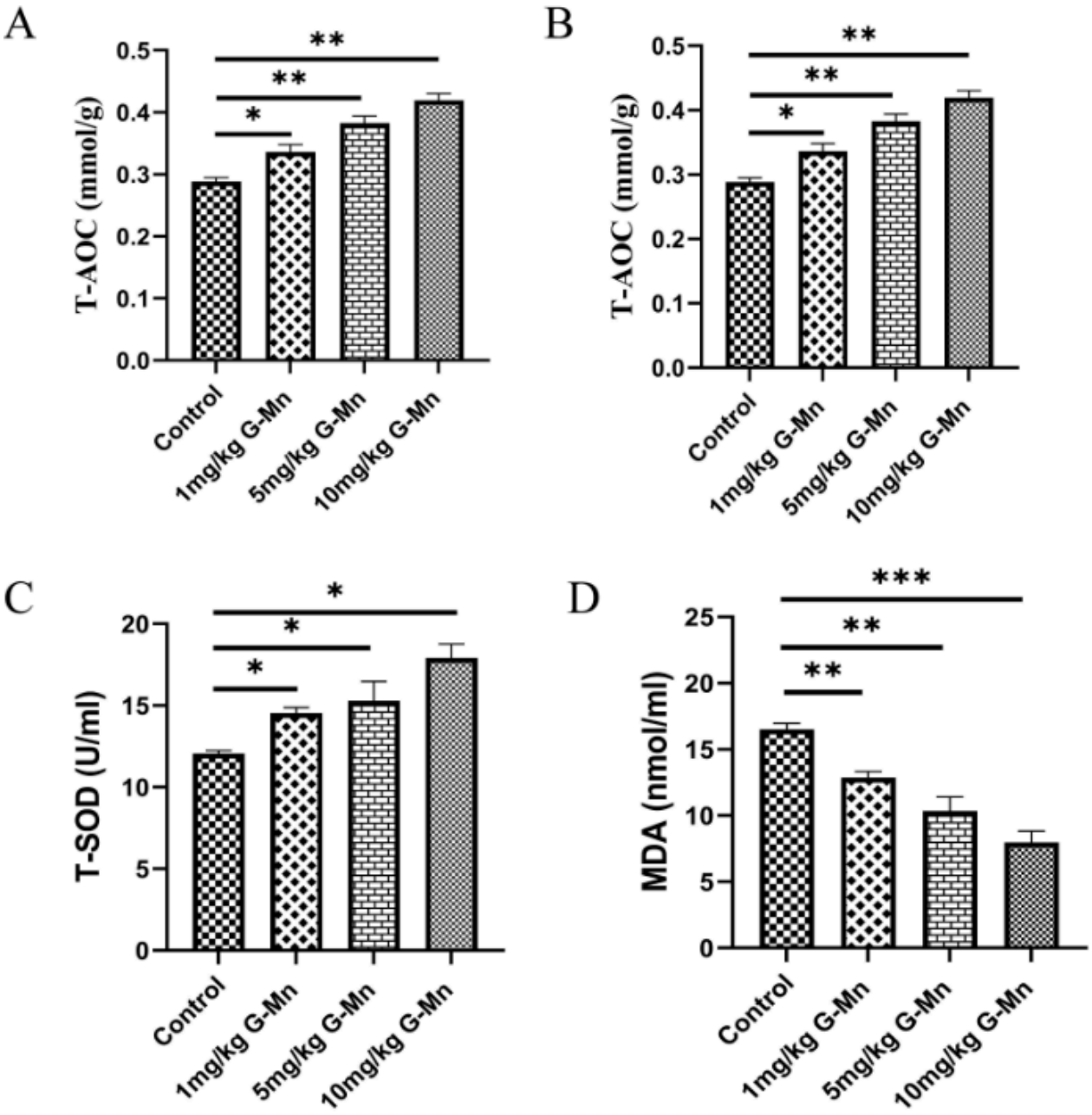


Figure 2

Effects of G-Mn supplementation on serum antioxidant status in broilers. (A) Total antioxidant capacity (T-AOC). (B) Glutathione peroxidase (GSH-PX) activity. (C) Total superoxide dismutase (T-SOD) activity. (D) Malondialdehyde (MDA) content. Data are presented as the mean ± standard error (SE). Asterisks indicate significant differences compared with the control group (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

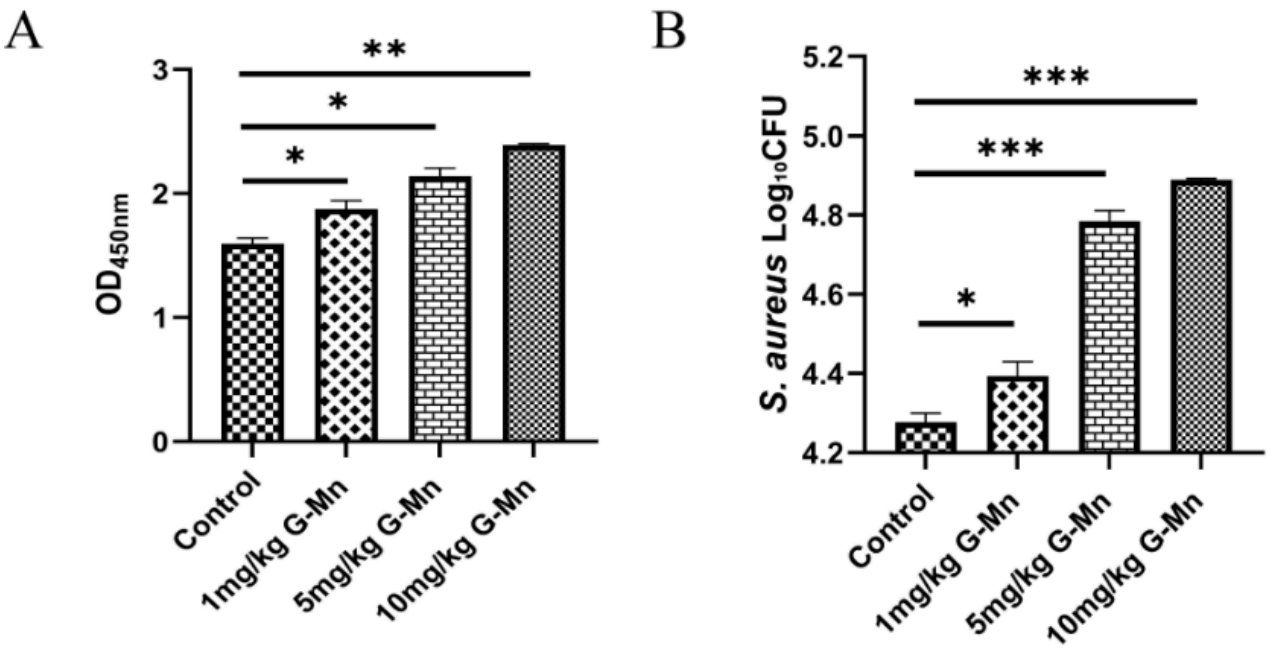


Figure 3

Effects of G-Mn supplementation on humoral immune response and antibody-mediated opsonophagocytosis in broilers. (A) Serum anti-*S. aureus* IgG levels, determined by indirect ELISA and expressed as absorbance at 450 nm (OD_{450}). (B) Antibody-mediated opsonophagocytic activity of serum against *S. aureus*, expressed as the recovered bacterial count (log_{10} CFU). Data are presented as the mean ± standard error (SE). Asterisks indicate significant differences compared with the control group (*P < 0.05, **P < 0.01, ***P < 0.001).

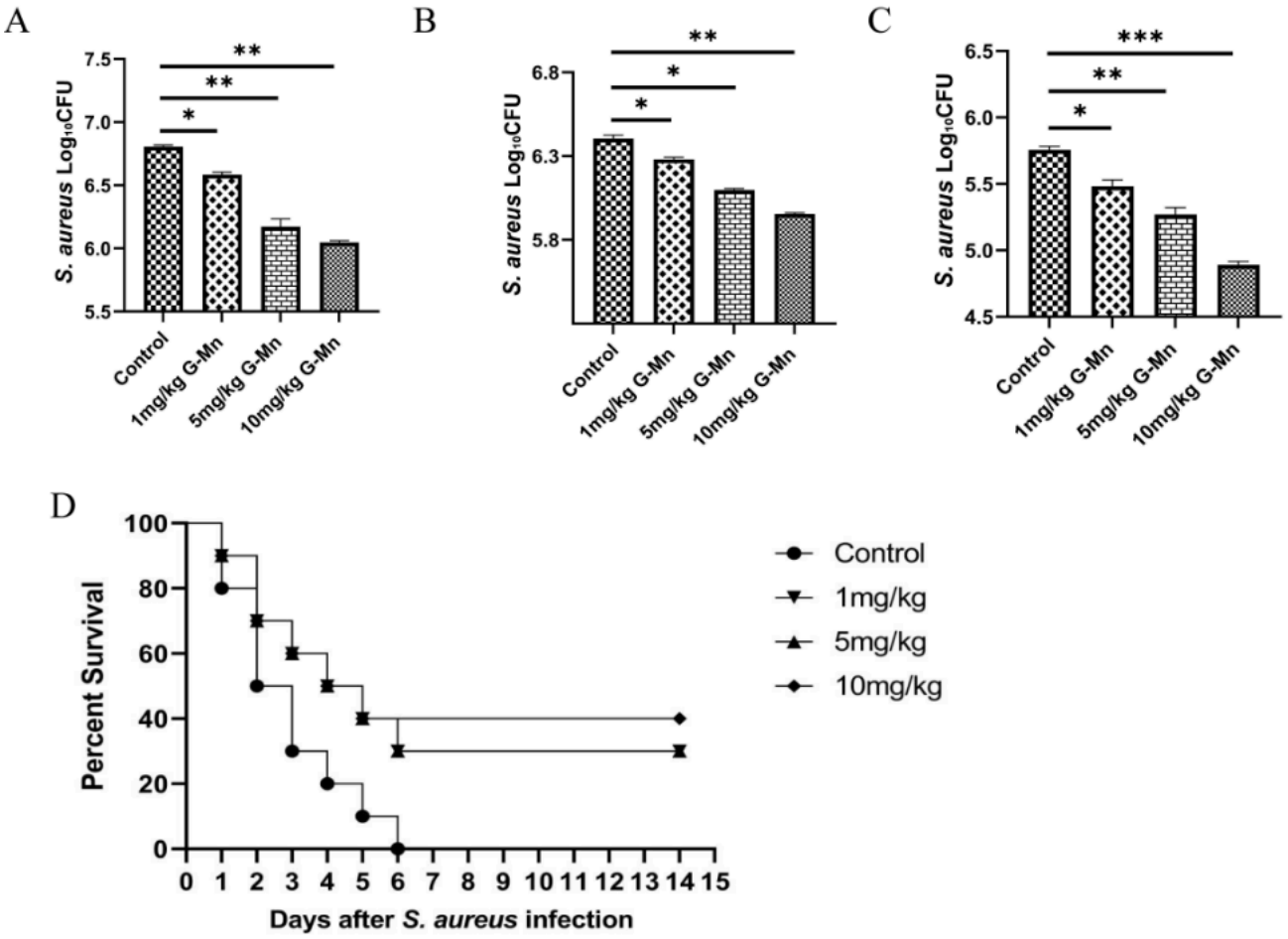


Figure 4

Effects of G-Mn supplementation on *S. aureus* colonization in target organs and survival rate of broilers after lethal challenge. (A-C) Bacterial loads in the liver (A), spleen (B), and kidney (C), expressed as $\log_{10}$ CFU/g of tissue. (D) Survival curves of broilers over a 14-day observation period following lethal *S. aureus* challenge. Data are presented as the mean ± standard error (SE). Asterisks indicate significant differences compared with the control group (*P< 0.05, **P < 0.01, ***P < 0.001).