Postprint: The Effect of Bacillus subtilis 048 on the Resistance of Xueshan Grass Chickens to Salmonella enteritidis Infection

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Abstract

This experiment aimed to investigate the effect of Bacillus subtilis (BS) 048 on the resistance of Snow Mountain grass chickens to Salmonella enteritidis (SE) infection. A total of 240 one-day-old Snow Mountain grass chicken roosters (SE-negative) with a body weight of (61.5$\pm$0.5)*gwererandomlydividedinto*4*groups*, *with*3*replicatespergroupand*2(
CFU daily during 5–7 days of age, and the experimental period was 14 days. The effects of BS048 on SE bacterial load in cecal mucosa, liver, and kidney, serum immunoglobulin (Ig) and ileal secretory immunoglobulin A (sIgA) contents, as well as jejunal mucosal alkaline phosphatase (ALP), myeloperoxidase (MPO), total superoxide dismutase (T-SOD), glutathione peroxidase (GSH-Px) activities, total antioxidant capacity (T-AOC), and malondialdehyde (MDA) content in normal and SE-infected Snow Mountain grass chickens were determined. The results showed: 1) Compared with the control group, the BS group significantly increased ileal sIgA content at 8, 10, and 14 days of age ($P<0.05$), significantly increased jejunal mucosal ALP activity at 10 and 14 days of age ($P<0.05$), extremely significantly increased jejunal mucosal T-AOC and T-SOD and GSH-Px activities at 10 and 14 days of age ($P<0.01$), and significantly decreased jejunal mucosal MDA content at 8 and 14 days of age ($P<0.05$). 2) Compared with the control infection group, the BS infection group extremely significantly reduced SE bacterial load in cecal mucosa, liver, and kidney of SE-infected Snow Mountain grass chickens ($P<0.01$), significantly increased serum IgA, IgG, IgM contents at 10 and 14 days of age and ileal sIgA content at 8, 10, and 14 days of age ($P<0.05$), significantly increased jejunal mucosal ALP activity at 8, 10, and 14 days of age $P<0.05$), extremely significantly decreased jejunal mucosal MPO activity at 8, 10, and 14 days of age ($P<0.01$), significantly or extremely significantly increased jejunal mucosal T-AOC and T-SOD and GSH-Px activities at 8, 10, and 14 days of age ($P<0.05$ or $P<0.01$), and significantly or extremely significantly decreased jejunal mucosal MDA content at 8, 10, and 14 days of age ($P<0.05$ or $P<0.01$). These results indicate

that BS048 can enhance the resistance of Snow Mountain grass chickens to SE infection.

Full Text

Effects of Bacillus subtilis 048 on Anti-Salmonella enteritis Infection Ability of Xueshan Chickens

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Abstract: This study investigated the effects of *Bacillus subtilis* 048 (BS048) on the anti-*Salmonella enteritis* (SE) infection ability of Xueshan chickens. Two hundred forty 1-day-old SE-negative Xueshan cockerels weighing (61.5$\pm$0.5)*gwererandomlyallocatedintofourgroupswiththreereplicatespergroupand*20*birdsperreplicate.The infectedgroupsreceivedabasaldiet*,*whiletheBSandBS*−*infectedgroupsreceivedthebasaldietsupplementedwi* CFU of SE daily from days 5 to 7. The 14-day trial evaluated BS048 effects on SE colonization in cecal mucosa, liver, and kidney; serum immunoglobulin (Ig) and ileal secretory IgA (sIgA) levels; and jejunal mucosal alkaline phosphatase (ALP), myeloperoxidase (MPO), total superoxide dismutase (T-SOD), glutathione peroxidase (GSH-Px) activities, total antioxidant capacity (T-AOC), and malondialdehyde (MDA) content.

The results showed: (1) Compared with the control group, BS supplementation significantly increased ileal sIgA content at 8, 10, and 14 days of age ($P<0.05$), significantly elevated jejunal mucosal ALP activity at 10 and 14 days ($P<0.05$), markedly enhanced T-AOC and T-SOD and GSH-Px activities at 10 and 14 days ($P<0.01$), and significantly reduced jejunal mucosal MDA content at 8 and 14 days ($P<0.05$). (2) Compared with the control-infected group, BS-infected group exhibited extremely significant reductions in SE load in cecal mucosa, liver, and kidney ($P<0.01$), significant increases in serum IgA, IgG, and IgM at 10 and 14 days and ileal sIgA at 8, 10, and 14 days ($P<0.05$), significantly higher jejunal mucosal ALP activity at 8, 10, and 14 days ($P<0.05$), markedly lower MPO activity at 8, 10, and 14 days ($P<0.01$), and significant or extremely significant improvements in T-AOC and T-SOD and GSH-Px activities at 8, 10, and 14 days ($P<0.05$ or $P<0.01$) along with significant or extremely significant reductions in MDA content ($P<0.05$ or $P<0.01$). These findings demonstrate that BS048 enhances the anti-SE infection capacity of Xueshan chickens.

Keywords: *Bacillus subtilis*; Xueshan chickens; *Salmonella enteritis*; anti-infection

Introduction

Salmonella is a Gram-negative, facultative anaerobic intracellular bacterium widely distributed in nature that can infect humans and various animals, with most serotypes exhibiting strong pathogenicity [1]. *Salmonella enteritis* (SE) causes enteritis in poultry, reduces production performance, and can lead to death. Contaminated poultry products further cause acute gastroenteritis in humans, posing serious threats to public health and food safety [2]. *Bacillus subtilis* (BS) is an aerobic Gram-positive bacterium that forms highly resistant endospores. As a high-quality, safe probiotic, BS improves animal growth performance, enhances immunity, exhibits antimicrobial activity, modulates intestinal microbiota, and boosts antioxidant function [3]. However, few studies have investigated BS effects on anti-*Salmonella* infection capacity and mechanisms in yellow-feathered broilers.

Xueshan chickens, a premium yellow-feathered broiler breed developed by Lihua Livestock Company researchers through crossbreeding Chinese indigenous breeds (Tibetan chickens and Chahua chickens), offer advantages including superior meat quality, strong disease resistance, simple management, and high economic returns. This study aimed to elucidate BS048 effects on anti-SE infection capacity and underlying mechanisms in Xueshan chickens, providing scientific evidence for novel BS preparation development and application in premium yellow-feathered broiler production.

Materials and Methods

Experimental Materials Two hundred forty 1-day-old Xueshan cockerels weighing $(61.5\$\pm0.5)g(SE-negative)wereobtainedfromJiangsuLihuaAnimalHusbandryCo.,Ltd.BS048prep$ CFU/g) was produced by Shandong Baolai-Lisheng Bioengineering Co., Ltd. The SE ATCC13076 strain was provided by Jiangsu Poultry Research Institute. ELISA kits for chicken IgA, IgG, and IgM were purchased from Life Diagnostics (USA). Assay kits for ALP, MPO, T-AOC, T-SOD, GSH-Px, and MDA were obtained from Nanjing Jiancheng Bioengineering Institute. Other routine reagents were analytical grade from Sangon Biotech (Shanghai). Main instruments included a MK3 microplate reader (Thermo, USA) and a UV-2100 UV-Vis spectrophotometer (Unico, Shanghai).

Experimental Design and Management Birds were randomly divided into four groups with three replicates per group (20 birds per replicate). The control and control-infected groups received basal diets, while the BS and BS-infected groups received basal diets supplemented with 0.1% (W/W) BS048. The control-infected and BS-infected groups were orally challenged with 1 mL SE suspension ($1\$\times10\hat{}\{8\}\$$ CFU/mL) daily from days 5–7 using gavage, while the control and BS groups received equivalent sterile saline. The trial lasted 14 days.

The basal diet was formulated according to Chinese Agricultural Industry Standard "Feeding Standard of Chicken"(NY/T 33—2004). Composition and nutrient

levels are shown in Table 1 . The trial was conducted at the experimental farm of Jiangsu Lihua Animal Husbandry Co., Ltd. using floor rearing with ad libitum feed and water. Lighting, vaccination, and other management followed conventional practices. Bird health and mortality were recorded daily.

Sample Collection and Measurements Samples were collected at 8, 10, and 14 days of age (1, 3, and 7 days post-challenge). Six birds per group (two per replicate) with similar body weight were selected for slaughter. Blood was collected from the heart, and after cervical dislocation, liver, kidney, and intestinal segments (jejunum, ileum, and cecum) were rapidly excised and stored at 4°C.

Tissue SE Load: Cecal mucosa, liver, and kidney samples were weighed and homogenized with 3 mL sterile PBS for 2 min on ice. Ten-fold serial dilutions were prepared, and 100 L of original and diluted samples were plated on XLT-4 agar. After incubation at 37°C for 24 h, typical SE colonies were counted.

Serum Immunoglobulins: Blood was coagulated at 37°C for 1 h, centrifuged at 1,000×g for 10 min, and serum IgA, IgG, and IgM levels were measured by ELISA according to kit instructions.

Ileal sIgA: A 10 cm ileal segment was flushed with 2 mL sterile saline. The flush fluid was collected, centrifuged at 5,000×g for 15 min at 4°C, and sIgA content in the supernatant was measured by ELISA.

ALP, MPO, and Antioxidant Indices: Approximately 0.1 g jejunal mucosa was homogenized with saline (1:9 W/V) on ice for 2 min, centrifuged at 1,500×g for 10 min, and supernatant ALP, MPO, T-SOD, GSH-Px activities, T-AOC, and MDA content were measured using Nanjing Jiancheng kits.

Statistical Analysis Data were analyzed using SPSS 22.0. Two-way ANOVA was performed using the GLM procedure, and one-way ANOVA with Duncan' s multiple comparison was applied. Significance was set at $P<0.05$, extreme significance at $P<0.01$, and trends at $0.05\leq P<0.10$. Results are expressed as means ± SD.

Results

SE Load in Cecal Mucosa, Liver, and Kidney As shown in Table 2 , BS-infected group exhibited extremely significant reductions in SE load in cecal mucosa at 8, 10, and 14 days compared with control-infected group ($P<0.01$). Similarly, SE loads in liver and kidney at 8 and 10 days were extremely significantly lower in BS-infected group ($P<0.01$).

These results demonstrate that continuous oral SE challenge for 3 days successfully established infection in cecal mucosa, liver, and kidney, with cecal loads substantially higher than hepatic and renal loads, confirming successful model establishment. Dietary BS048 supplementation significantly reduced SE

colonization in these tissues, indicating protective effects during SE infection, consistent with findings from Wang et al. [4] and Xiang et al. [5].

Serum Immunoglobulin and Ileal sIgA Contents Table 3 shows that BS treatment significantly affected serum IgA, IgG, IgM, and ileal sIgA at 10 and 14 days ($P<0.05$), while SE infection significantly impacted serum IgA (days 7, 10, 14), IgG (days 7, 14), IgM (day 7), and sIgA (days 7, 10, 14) ($P<0.05$). No significant BS × SE interactions were observed for these parameters ($P>0.05$).

Compared with controls, BS group showed no significant changes in serum immunoglobulins ($P>0.05$) but significantly increased ileal sIgA by 28.41% and 29.24% at 10 and 14 days ($P<0.05$). Control-infected group exhibited significant reductions in serum IgA at 10 and 14 days (24.80% and 41.68%, $P<0.01$) and IgG at 8 days (20.29%, $P<0.05$), but significant increases in IgG at 14 days (56.53%, $P<0.01$) and IgM at 8 days (21.34%, $P<0.01$). BS-infected group showed decreased serum IgG at 8 days (17.55%, $P<0.05$) but increased IgG at 10 and 14 days (25.34% and 96.16%, $P<0.05$ and $P<0.01$) and IgM at 8 days (26.72%, $P<0.01$). Compared with control-infected group, BS-infected group significantly increased serum IgA, IgG, and IgM at 10 and 14 days by 18.70%, 47.61%, 29.36%, 25.32%, 27.36%, and 19.11% ($P<0.05$), respectively.

For ileal sIgA, BS group showed significant increases at 10 and 14 days ($P<0.05$). Control-infected group had reduced sIgA at 8 days (32.50%, $P<0.05$) but increased sIgA at 14 days (33.24%, $P<0.05$). BS-infected group significantly increased sIgA at 8, 10, and 14 days compared with control-infected group (41.97%, 40.22%, and 27.24%, $P<0.05$).

These findings indicate that BS048 supplementation enhances intestinal mucosal immunity by promoting sIgA secretion without affecting serum immunoglobulins in healthy birds. SE infection triggers both humoral and mucosal immune responses, while BS048 significantly boosts both immunoglobulin and sIgA secretion during SE infection, enhancing specific immunity and SE clearance.

Jejunal Mucosal ALP and MPO Activities Table 4 reveals that BS treatment and SE infection significantly affected jejunal ALP and MPO activities at 8, 10, and 14 days ($P<0.05$). No significant BS × SE interaction was observed for ALP ($P>0.05$), but a significant interaction existed for MPO ($P<0.05$).

BS group showed increased ALP activity at 14 days (37.26%, $P<0.05$) with no significant MPO changes ($P>0.05$). Control-infected group exhibited significant or extremely significant ALP reductions at 8, 10, and 14 days (61.71%, 55.07%, and 42.22%, $P<0.01$ and $P<0.05$) and MPO increases (291.92%, 294.24%, and 234.23%, $P<0.01$). BS-infected group showed decreased ALP at 8 days (25.61%, $P<0.05$) but increased MPO at 8, 10, and 14 days (98.28%, 77.58%, and 41.92%, $P<0.01$ and $P<0.05$). Compared with control-infected group, BS-infected group significantly increased ALP at 8, 10, and 14 days (94.29%, 101.32%, and 74.45%, $P<0.05$) and extremely significantly decreased MPO (49.41%, 54.96%, and

57.54%, P<0.01).

These results demonstrate that BS048 enhances intestinal barrier function by increasing ALP activity without inducing inflammation in healthy birds. SE infection impairs the intestinal barrier (reduced ALP) and triggers inflammation (elevated MPO). BS048 protects the intestinal mucosa during SE infection by restoring ALP activity and reducing MPO activity, consistent with Li et al. [8].

Jejunal Mucosal Antioxidant Function Table 5 shows BS treatment significantly affected T-AOC, T-SOD, MDA at 8, 10, 14 days and GSH-Px at 10, 14 days (P<0.05). SE infection significantly impacted all antioxidant parameters at 7, 10, and 14 days (P<0.05). No significant BS × SE interactions were observed (P>0.05).

BS group exhibited significant or extremely significant increases in T-AOC and T-SOD and GSH-Px at 10 and 14 days (37.20%, 43.00%, 35.83%, 72.52%, 43.81%, and 74.52%, P<0.01 and P<0.05) and MDA reductions at 8 and 14 days (38.28% and 40.47%, P<0.05). Control-infected group showed significant or extremely significant decreases in T-AOC and T-SOD and GSH-Px at 8, 10, and 14 days (48.60%, 20.55%, 28.31%, 28.08%, 34.07%, 33.04%, 48.96%, 35.79%, and 36.00%, P<0.01 and P<0.05) and MDA increases (33.95%, 88.02%, and 33.63%, P<0.05 and P<0.01). BS-infected group showed increased T-SOD at 14 days (35.68%, P<0.05). Compared with control-infected group, BS-infected group significantly or extremely significantly improved T-AOC and T-SOD and GSH-Px at 8, 10, and 14 days (62.91%, 22.63%, 51.09%, 32.56%, 64.60%, 102.63%, 81.96%, 49.68%, and 85.33%, P<0.05 and P<0.01) and reduced MDA (28.88%, 38.64%, and 27.73%, P<0.05 and P<0.01).

These findings indicate BS048 significantly enhances intestinal antioxidant function in healthy birds. SE infection causes oxidative stress and impairs antioxidant capacity, while BS048 alleviates oxidative damage and restores antioxidant function during infection, consistent with Gong et al. [10].

Discussion

The successful establishment of the SE infection model was confirmed by high SE loads in cecal mucosa, liver, and kidney after 3-day oral challenge, with cecal loads substantially exceeding hepatic and renal loads. Dietary BS048 significantly reduced SE colonization across all tissues, demonstrating protective effects during infection that align with previous research [4,5].

Serum IgG and intestinal sIgA are crucial components of specific immunity, playing key roles in humoral and mucosal immunity, respectively. BS048 supplementation significantly promoted intestinal sIgA secretion in healthy birds, enhancing mucosal anti-infection capacity. SE infection triggered both humoral and mucosal immune responses, evidenced by immunoglobulin consumption and production. BS048 significantly boosted both serum immunoglobulin and intestinal sIgA secretion during SE infection, enhancing specific immunity and

SE clearance capacity, which corresponds with the reduced tissue SE loads observed.

ALP is a ubiquitous phosphomonoester hydrolase with four isoenzymes: intestinal, placental, germ cell, and tissue-nonspecific (liver, bone, kidney) [6]. Intestinal ALP maintains intestinal barrier integrity by regulating duodenal surface pH, detoxifying lipopolysaccharide and free nucleotides, suppressing inflammation, and modulating microbiota [7]. MPO, a marker of myeloid cells (neutrophils and monocytes), indicates neutrophil infiltration and tissue inflammation. BS048 increased jejunal ALP activity without affecting MPO in healthy birds, suggesting enhanced barrier function without mucosal damage. SE infection reduced ALP and increased MPO, indicating barrier disruption and neutrophil infiltration. BS048 restored ALP activity and reduced MPO during SE infection, demonstrating protective effects against mucosal damage and inflammation, consistent with Li et al. [8].

Animals possess sophisticated antioxidant systems to prevent free radical accumulation and oxidative damage. BS048 enhanced intestinal antioxidant function in healthy birds, similar to Li et al. [9]. Pathogen invasion triggers stress responses generating reactive oxygen/nitrogen species and inflammatory factors to combat pathogens, but this causes oxidative and inflammatory tissue damage affecting normal physiological functions. Gong et al. [10] reported that SE infection reduces broiler antioxidant function, while dietary *Bacillus coagulans* alleviates oxidative stress through enhanced antioxidant capacity, consistent with our findings.

In conclusion, BS048 enhances anti-SE infection capacity in Xueshan chickens through three mechanisms: (1) enhancing humoral and mucosal immunity to promote SE clearance; (2) strengthening intestinal barrier function to reduce infection-induced damage and inflammation; and (3) improving intestinal antioxidant function to alleviate bacterial infection-induced oxidative stress and protect tissue function.

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Note: Figure translations are in progress. See original paper for figures.