Postprint of Safety Evaluation of Enterococcus faecium WEI-10 in Weaned Piglets

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Abstract

This study evaluated the safety of strain WEI-10 for weaned piglets by supplementing Enterococcus faecium WEI-10 in the diet and assessing activity status, diarrhea scores, growth performance, bacteremia, and histopathology. Two batches of experiments were conducted, each using 253 weaned piglets at 26 days of age with average body weights of $(7.44\$\pm1.02)and(8.11\pm1.51)kg, respectively.Eachbatchwasdividedinto2groups$: $thecontrolgroupwasfedabasaldiet(withoutantibioticsorprobiotics), andthetreatmentgroupwasfedatestdietsu$ $10inthebasaldiet(thesupplementationlevelsinthetreatmentgroupsofthe2batcheswere1\times108CFUand1\times\$107$ CFU per gram of basal diet, respectively). Each batch lasted 38 days. The results showed: 1) In both batches, there were no significant differences between the treatment and control groups in activity scores, average daily gain, and feed-to-gain ratio ($P>0.05$). 2) The diarrhea scores of the treatment group were significantly lower than those of the control group on days 5, 10, and 38 ($P<0.05$); on day 38, the diarrhea score of the treatment group decreased to 0, while that of the control group was approximately 0.6. 3) Neither the treatment nor control groups in the two batches exhibited bacteremia or pathological changes in the heart, liver, spleen, lung, kidney, duodenum, or jejunum. These results indicate that strain WEI-10 poses no safety concerns for weaned piglets and can reduce diarrhea.

Full Text

Safety Evaluation of *Enterococcus faecium* WEI-10 on Weaned Piglets

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Abstract: This study evaluated the safety of *Enterococcus faecium* WEI-10 for weaned piglets by supplementing it in the diet and examining activity status, diarrhea scores, growth performance, bacteremia, and histopathological changes. Two batch experiments were designed. In each batch, 253 crossbred (Large White × Landrace × Duroc) weaned piglets at 26 days of age with average body weights of $(7.44\$\pm1.02)kgand(8.11\pm1.51)kg, respectively, wererandomlyassignedtotwogroups:$ $acontrolgroupfedabasaldietwithoutantibioticsorprobiotics, andatestgroupfedthebasaldietsupplementedwith$ $E.faecium*WEI-10atdosesof1\times10\hat{}\{8\}\$$ CFU/g and $1\$\times10\hat{}\{7\}\$$ CFU/g in the two batches, respectively. Each experiment lasted 38 days. The results showed: 1) No significant differences were observed between test and control groups in activity scores, average daily gain, or feed-to-gain ratio in either batch ($P>0.05$). 2) Diarrhea scores in test groups were significantly lower than those in control groups on days 5, 10, and 38 ($P<0.05$); on day 38, diarrhea scores in test groups dropped to 0, while control groups maintained scores around 0.6. 3) Neither test nor control groups in either batch exhibited bacteremia or pathological changes in the heart, liver, spleen, lung, kidney, duodenum, or jejunum. These findings indicate that strain WEI-10 poses no safety concerns for weaned piglets and can effectively reduce diarrhea.

Keywords: *Enterococcus faecium*; weaned piglets; safety; growth performance; bacteremia; histopathology

Introduction

Enterococci are part of the normal intestinal flora in humans and animals. Previously considered harmless commensal bacteria, recent studies have confirmed that certain species and strains within the *Enterococcus* genus possess pathogenic potential for both humans and animals. The number of infection cases caused by these strains has been increasing, making them common opportunistic pathogens in hospital-acquired infections. Furthermore, mounting evidence has linked *Enterococcus faecium* to clinical pathologies such as bacteremia, infectious endocarditis, and abscesses [1].

Although probiotic safety evaluation has been a major research focus in recent decades, most studies on *E. faecium* safety have relied on in vitro assays, including hemolysis, virulence genes, antibiotic resistance, and toxic metabolite production. However, in vitro safety evaluation results often fail to accurately reflect in vivo effects. Currently, in vivo safety evaluation studies are scarce and unsystematic, and no standardized guidelines for in vivo probiotic safety assessment have been established. Current preclinical in vivo safety evaluation of food-grade probiotics includes acute and subchronic toxicity tests. Key indicators in subchronic toxicity tests include animal activity status, diarrhea scores (toxicity clinical symptoms), and growth performance [2-3], which have been widely adopted in safety evaluation studies [4-5]. Bacterial translocation

is another recommended safety assessment indicator [6-7], as it represents the first step in pathogenesis for many endogenous opportunistic pathogens. Hepatosplenomegaly and other organ lesions serve as indirect indicators of infectivity and pathogenicity. Intestinal mucosal integrity plays a crucial barrier role in preventing invasion by potential pathogens or toxic substances into systemic tissues, making it another important safety evaluation metric [8].

Both literature reports and market practices demonstrate that the daily dosage required for probiotics to exert beneficial effects in livestock is 10^6 CFU per gram of basal diet [9-10]. This study selected doses of 10× and 100× the normal daily dosage (i.e., 1$×10^{7}$ CFU/g and 1$×10^{8}$ CFU/g) of a laboratory-isolated *E. faecium* strain WEI-10 supplemented in weaned piglet diets. Through comprehensive investigation of piglet activity status, diarrhea scores, growth performance, bacteremia, and histopathology, this research conducted a systematic in vivo safety evaluation of strain WEI-10 specifically for weaned piglets, aiming to assess the in vivo safety of feed-grade *E. faecium*.

Materials and Methods

1.1 Strain and Test Material

The strain used in this study was *Enterococcus faecium* WEI-10 (China General Microbiological Culture Collection Center No. 7746), isolated from the intestinal mucosa of healthy 60-day-old piglets. Previous studies have evaluated its in vitro safety and efficacy [11] and conducted acute and subacute toxicity tests in mice [3]. In vitro safety tests revealed that strain WEI-10 exhibited γ-hemolysis, lacked bile salt hydrolase activity, did not produce biogenic amines (histamine, tyramine, or putrescine), contained none of the three virulence genes (IS16, esp, and hylEFM) required for detection under the European Food Safety Authority (EFSA) 2012 guidelines for *E. faecium* virulence factor evaluation [12], and harbored no transferable antibiotic resistance genes. Acute and subacute toxicity tests in mice showed that neither the original fermentation broth (3$×10^{9}$ CFU/mL) nor the raw powder (3$×10^{10}$ CFU/g) caused mortality, weight loss, or significant differences in fur appearance, activity status, feed intake, or organ indices of liver, spleen, lung, and kidney compared with the control group ($P>0.05$). Histopathological examination revealed no substantive lesions in these organs. The test material used in this study was a granulated powder produced in our laboratory with a viable count of 10^{10} CFU/g.

1.2 Experimental Animals and Design

The experiment was conducted at a commercial pig farm in Harbin, Heilongjiang Province. A single-factor completely randomized design was employed with two batch experiments. In Batch 1, 253 healthy 26-day-old weaned piglets (Landrace × Large White × Duroc crossbreed) with similar body condition and average weight of (7.44$±1.02)*kgwereselectedandrandomlydividedintotwogroupsaccordingtosimilarbodyweightandba*
ControlGroupIwasfedabasaldietwithoutantibioticsorprobiotics, *andTestGroupIwasfedthebasaldietsupplem*

$10at1\times10\hat{}\{8\}\$$ CFU/g. In Batch 2, another 253 healthy 26-day-old weaned piglets with average weight of $(8.11\$\pm1.51)kgwererandomlydividedintoControlGroupIIandTestGroupII,wit$ $10at1\times10\hat{}\{7\}\$$ CFU/g. Each batch experiment lasted 38 days.

1.3 Organ Index Measurement

One piglet near the average body weight was selected from each replicate, euthanized by cardiac exsanguination, and immediately necropsied. The heart, liver, spleen, lung, and kidney were collected, adipose tissue was removed, and wet weights were measured to calculate organ indexes.

Organ index (g/kg) = organ wet weight (g) / live body weight (kg).

1.4 Histopathological Examination

After ligating the gastrointestinal segments, 2 cm sections were taken from the proximal duodenum and jejunum. Two samples each of heart, liver, spleen, lung, and kidney were collected and fixed in 10% formalin, followed by gradient ethanol dehydration, xylene clearing, and paraffin embedding. For intestinal sections, 3-6 transverse sections of intestinal rings were continuously cut from 3-6 sites in both duodenal and jejunal segments. For each organ sample, 3 transverse or longitudinal sections were continuously cut from 3 sites. Paraffin sections of 4-6 m thickness were prepared and stained with hematoxylin-eosin (HE). Basic morphology and pathological changes in intestinal and organ tissues were observed under an optical microscope, and images were captured using an image acquisition system.

1.5 Data Analysis

All experimental data are expressed as mean ± standard deviation. One-way ANOVA was performed using SPSS 16.0 statistical software, with $P<0.05$ considered statistically significant.

Results

2.1 Effects of *E. faecium* WEI-10 on Activity Status of Weaned Piglets

Activity status was assessed using activity scores. As shown in Figure 1 [Figure 1: see original paper], throughout the experimental period, activity scores were 2.22 and 2.15 for Control Group I and Test Group I, respectively, in Batch 1, and 2.30 and 2.35 for Control Group II and Test Group II, respectively, in Batch 2. These scores fell between intermediate status and active movement/foraging. No significant differences in activity status were observed between test and control groups in either batch ($P>0.05$), and no mortality or disease occurred in any group.

2.2 Effects of *E. faecium* WEI-10 on Diarrhea Scores of Weaned Piglets

Diarrhea scores for the two batch experiments are shown in Figure 2 [Figure 2: see original paper]. Significant differences between test and control groups were observed on days 5, 10, and 38 ($P<0.05$). Throughout the feeding period, test groups in both batches had lower diarrhea scores than control groups. By day 38, diarrhea scores in test groups decreased to 0, while control groups maintained scores around 0.6. Therefore, even at 10× and 100× the normal dosage, *E. faecium* WEI-10 did not cause diarrhea in weaned piglets but instead reduced diarrhea scores.

2.3 Bacteremia Detection

Bacteremia was not detected in any piglet from either test or control groups in both batches, indicating that strain WEI-10 did not cause bacterial translocation from the intestine to systemic circulation.

2.4 Effects of *E. faecium* WEI-10 on Organ Indexes of Weaned Piglets

Table 3 presents the organ index results. In Batch 1, organ indexes for heart, liver, spleen, lung, and kidney were $5.801\$\pm0.854, 31.436\pm2.116, 2.873\pm0.512, 14.033\pm1.229, and 4.254\pm0.742 g/$ g/kg in Control Group II. No significant differences in organ indexes were observed between test and control groups in either batch ($P>0.05$).

2.5 Effects of *E. faecium* WEI-10 on Histopathological Changes

Histopathological analysis revealed no substantive organ lesions, increased inflammatory cells, corresponding lymphocytes, or structural loosening and edema in test groups. Strain WEI-10 feeding did not affect intestinal mucosal integrity in the duodenum and jejunum, nor did it cause mucosal epithelial cell shedding, exposed lamina propria, lamina propria loosening, or inflammatory reactions due to lymphocyte infiltration.

Using liver, spleen, and duodenum as examples, histopathological results from both batches were analyzed. As shown in Figures 3 [Figure 3: see original paper], 4 [Figure 4: see original paper], and 5 [Figure 5: see original paper], liver tissues in both control and test groups appeared normal, with distinguishable hepatic lobule structure and orderly hepatic cord arrangement, showing no pathological features such as hepatocyte vacuolation, hepatic sinusoid dilation, or lymphocyte/inflammatory cell infiltration. Spleen tissues in both groups were normal, with clear marginal zones between white and red pulp, no white pulp hemorrhage, distinct red pulp splenic cords, and no splenic sinus dilation. Duodenal tissues showed no mucosal epithelial cell shedding, exposed lamina propria, edema, loosening between lamina propria and muscularis mucosae/submucosa, or eosinophil and plasma cell infiltration.

Abbreviations: C1: Control Group I; T1: Test Group I; C2: Control Group II; T2: Test Group II. The same applies to the following figures.

Discussion

3.1 Effects of *E. faecium* WEI-10 on Overall Activity Status, Diarrhea Scores, and Growth Performance of Weaned Piglets

Appetite, activity status, diarrhea condition, and growth performance are sensitive indicators of animal health status. This study evaluated the in vivo safety of *E. faecium* WEI-10 for weaned piglets using average daily feed intake, average daily gain, feed-to-gain ratio, activity scores, and diarrhea scores as metrics. The selected dosages were 1$×10^{7}$ CFU/g and 1$×10^{8}$ CFU/g (10× and 100× the normal daily dosage). Although test groups in both batches showed slightly lower average daily feed intake and average daily gain, and Batch 1 test group had a slightly higher feed-to-gain ratio compared with their respective control groups, no significant differences were observed between test and control groups in either batch. Therefore, strain WEI-10 was preliminarily determined to pose no health threat to weaned piglets. Similar results were reported by Hanczakowska et al. [19] in studying the effects of *E. faecium* NCIMB 10415 on growth performance of 26-56 day-old piglets, where the test group showed lower average daily feed intake and average daily gain and higher feed-to-gain ratio than the control group, but without significant differences. Zhou et al. [4] evaluated the toxicity of *Lactobacillus rhamnosus* HN001, *Lactobacillus acidophilus* HN017, and *Bifidobacterium lactis* HN019 and found no significant differences in activity scores compared with control groups and commercial strains *L. rhamnosus* GG and *L. acidophilus* LA-1.

This study demonstrated that test groups in both batches had reduced diarrhea scores compared with control groups, consistent with previous research. Chen et al. [22] reported that dietary probiotic supplementation reduced ammonia concentration in pig excreta, thereby decreasing diarrhea scores. Thu et al. [23] found that adding 3% liquid metabolites of *Lactobacillus plantarum* to weaned piglet diets significantly reduced diarrhea scores. Hua et al. [17] reported that adding 6% *E. faecium* T013 compound preparation to weaned piglet diets significantly reduced diarrhea incidence.

3.2 Bacteremia Detection Results in Weaned Piglets

Bacterial translocation or bacteremia is a prerequisite for pathogenesis in most intestinal opportunistic pathogens and serves as an important indicator of bacterial infectivity and pathogenicity [24]. Strain WEI-10 was not detected in the blood of piglets in this study, indicating that it did not translocate from the intestine to systemic blood tissues and poses no translocation risk. Similar conclusions were drawn by Zhou et al. [4] in toxicity evaluations of *L. rhamnosus* HN001, *L. acidophilus* HN017, and *B. lactis* HN019. Therefore, strain WEI-10 is considered non-invasive and safe for piglets.

3.3 Effects of *E. faecium* WEI-10 on Organ Indexes and Major Histopathological Changes

Infectivity and pathogenicity are also important indicators for probiotic safety evaluation. Hepatosplenomegaly serves as an indirect indicator of infectivity. Necropsy observations revealed no substantive lesions or obvious differences in liver, spleen, or other organs between test and control groups in either batch, and measured organ indexes showed no significant differences between groups. Peripheral blood neutrophils or eosinophils are effective indicators of bacterial infection. Histopathological analysis detected no substantive organ lesions, increased inflammatory cells, corresponding lymphocytes, structural loosening, or edema in test groups, with no obvious pathological differences from control groups, indicating that strain WEI-10 did not cause infection or disease in piglets. Intestinal mucosal integrity plays a vital barrier role in preventing invasion by potential pathogens or toxic substances into systemic tissues [8]. Strain WEI-10 in this study did not adversely affect duodenal or jejunal mucosal integrity in test group piglets, nor did it cause mucosal epithelial cell shedding, exposed lamina propria, lamina propria loosening, or inflammatory reactions from lymphocyte infiltration, demonstrating that strain WEI-10 lacks infectivity and pathogenicity. Similar conclusions were reported by Giannenas et al. [21] in evaluating the effects of *E. faecium* on intestinal mucosa of fattening pigs.

Conclusions

Based on the results of this study, the following conclusions can be drawn: (1) Even at 10× and 100× the normal daily dosage, *E. faecium* WEI-10 administration to weaned piglets caused no significant changes in any measured indicators (activity scores, diarrhea scores, average daily feed intake, average daily gain, and feed-to-gain ratio), although some parameters showed slight deterioration. (2) Strain WEI-10 did not cause translocation to extraintestinal tissues or histological lesions in internal organs or intestinal mucosa of weaned piglets. (3) *Enterococcus faecium* WEI-10 poses no safety concerns.

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