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APPLIED MICROBIAL AND CELL PHYSIOLOGY

Bacillus strains improve growth performance via enhancing digestive function and anti-disease ability in young and weaning rex rabbits

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Abstract

Numerous studies have shown that probiotic *Bacillus* could promote growth and enhance anti-disease ability in animal. In present study, the mixture of three *Bacillus* strains, which were isolated from rex rabbits and showed high cellulose, protease, and amylase activities, was added into the diet for investigating its effects on young and weaning rex rabbits. For experiment 1, 40 young rex rabbits (9 weeks old) were randomly divided into four groups and fed with diets containing 0 (NC), 1.0×10^5 cfu/g (LC), 1.0×10^6 cfu/g (MC), and 1.0×10^7 cfu/g (HC) *Bacillus* strains for 4 weeks. For experiment 2, 80 weaning rex rabbits (5 weeks old) were randomly divided into four groups and fed with diet containing 0 (control), 1.0×10^5 cfu/g (T-1), 1.0×10^6 cfu/g (T-2), and 1.0×10^7 cfu/g (T-3) *Bacillus* strains for 8 weeks. The results showed that *Bacillus* strains at a dose of 1.0×10^6 cfu/g significantly enhanced growth performance, increased immune organ indexes, improved serum biochemical parameters, and heightened antioxidant capacity. It also markedly improved the intestinal microbiota by increasing *Lactobacillus* spp., *Bacillus* spp. counts, and decreased *Escherichia coli* count. In addition, the *Bacillus* mixture raised the concentrations of acetic acid, propionic acid, and butyric acid as well as protease, amylase, and cellulase activities of young and weaning rex rabbits. Moreover, for weaning rex rabbits, the inclusion of *Bacillus* strains also upregulated the abundance of cellulolytic bacteria and improved intestinal morphology. Therefore, our results indicated that *Bacillus* strains could facilitate the growth of young and weaning rex rabbits by improving digestive function and anti-disease ability.

Key Points

- *Bacillus with high extracellular enzyme activity were isolated from rex rabbits.*
- *Bacillus could improve growth performance of young and weaning rex rabbits.*
- *The digestive function of young and weaning rex rabbits could be improved by Bacillus.*

Keywords *Bacillus* · Growth performance · Digestive function · Anti-disease ability · Young and weaning rex rabbits

Jie Wang, Xueqin Ni, and Bin Wen are joint first authors.

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Introduction

Considering that rex rabbits serve as suppliers of crucial raw materials for superior fur production as well as the sources of high-quality meat products (Liu et al. 2019), the demand of rex rabbits is increasing every year. Cellulose, which plays an important role on health, is a major component of rabbits' diet and mainly decomposed by cecum microbiota. However, the digestibility of cellulose remains inefficient (Gidenne et al. 2000). The incomplete development of digestive system in young and weaning rabbits is often accompanied with insufficient secretion of gastric acid and digestive enzymes, leading to diarrhea and

other intestinal diseases which could be considered as a key limiting factor for the development of rabbit breeding industry (Kupczyński et al. 2016; Zhou et al. 2018). The abuse of antibiotics as antimicrobial growth promoters (AGPs) or drugs has brought many side effects including intestinal microbiota disorder and inflammation of parents and their offsprings. It also causes the alteration of metabolic homeostasis and immune response, resulting in increasing susceptibility of various diseases (Willing et al. 2011; Zarrinpar et al. 2018).

Probiotics have been used worldwide and are considered to be an alternative of antibiotics (Yu et al. 2015). Due to the characteristics of host specificity, the adhesion and beneficial effects of probiotics are often greater when they are obtained from the same animal speices (Anderson and Gilliland 1999). For example, a current finding demonstrated that the effect of a turkey-derived probiotic on enhancing turkeys' performance metrics was significantly higher than probiotic derived from other poultry via modulating microbiome, mycobiome, and host gene expression (Ward et al. 2019). Another latest study also mentioned that dietary supplementation with pig-derived *Clostridium butyricum* could cause diarrhea in weaning rex rabbits because of high sensitivity of the gastrointestinal tract (Liu et al. 2019). Owing to acid resistance, bile salt resistance, and viability during distribution and storage, *Bacillus* strains are considered a potential commercial strain (Cartman et al. 2008). They can produce a variety of extracellular enzymes to accelerate the digestion and absorption of nutrients, including amylase, cellulase, and protease (Lee et al. 2008). Numerous studies showed that *Bacillus*-based preparations could promote growth performance (Lee et al. 2014) and improve immunity and micro-ecological balance (Hu et al. 2014) as well as reduce oxidative damages (Zhang et al. 2013).

In our study, we separated *Bacillus* strains from cecal content of healthy rex rabbits and screened *Bacillus* strains with high abilities to produce protease, cellulase, and amylase. We subseqently assessed the effects of a mixture of three isolated *Bacillus* strains on the health of young and weaning rex rabbits. Besides growth performance, organ indexes, biochemical parameters, and antioxidant indexes in the serum were tested in the present study. We also determined intestinal microbiota, volatile fatty acid (VFA) concentration, digestive enzyme activities, and intestinal morphology.

Materials and methods

Isolation, screening, and identification of *Bacillus* strains

Cecum contents of healthy rex rabbits were obtained from Sichuan Academy of Grassland Science (Chengdu, China). Cecum content (0.5 g) suspended in 49.5 mL of sterile saline in a conical flask. After pretreatment at 80 °C for 30 min, mixed liquor was diluted and spread onto nutrient agar (NA) plates and cultured for 24 h in a 37 °C incubator. And then, suspected *Bacillus* were picked for next research. These strains were screened based on cellulose, protease, and amylase activities. Preliminary enzyme assays were performed correspondingly using carboxy methyl cellulose (CMC), casein, and starch agar medium. The diameter of the bacterial colony (C) and the hydrolysis zone (H) were measured to calculate relative enzyme activity (REA = H/C) and those with higher REA were chosen for further enzymatic activity assay. Further enzyme assays were carried out using modified dinitrosalicylic acid (DNS), Folin reagent, and DNS method, respectively (Eveleigh et al. 2009; Sugiura et al. 1979). One unit (U) of carboxy methyl cellulase (CMC-ase)/filter paperase (FP-ase)/protease/amylase activity was defined as the amount of enzyme that release 1 μmole of glucose/glucose/tyrosine/maltose from CMC/filter paper (FP)/casein/starch per mL per min. Strains with excellent enzymatic activities in top three for each of the evaluated enzymes were selected and a three-factor and three-level orthogonal experiment was designed (Table S1). The proportion of three *Bacillus* strain cells in each combination was 1:1:1. When selecting the optimal combination, cellulase activity was given priority followed by protease and amylase activity. Biochemical tubes were used for biochemical identification in accordance with Bergey's Manual of Systematic Bacteriology. Subsequently, 16S rDNA gene sequencing was conducted using general primers 1492R and 27F, and the DNA sequence was blasted in GenBank (http://www.ncbi.nlm.nih.gov).

Experimental design

The powders of *Bacillus* cells BSWJ2017001, BSWJ2017002, and BSWJ2017003 were separately prepared by the Laboratory of Animal Microecology, Sichuan Agricultural University (Chengdu, China) and then mixed in a ratio of 1:1:1. The mixture was intermingled with premix before feed granulation.

Prior to the beginning of each experiment, rex rabbits were kept in separated cages for 1 week to get used to the environment. Every group was half males and half females. For animal experiment 1, 40 9-week-old young Sichuan white rex rabbits (1.93 ± 0.15 kg average weight) were randomly separated into four treatments with 5 replicates (2 rabbits per replicate): normal control (NC) group, low-dose control (LC) group, middle-dose control (MC) group, and high-dose control (HC) group fed with a basal diet supplemented with 0, 1.0×10^5, 1.0×10^6, and 1.0×10^7 cfu/g *Bacillus* strains for 4 weeks, respectively. For animal experiment 2, a total of 120 weaning (5 weeks) Sichuan white rex rabbit with similar body weight (1.08 ± 0.13 kg) were assigned to four groups at random with 5 replicates (6 rabbits per replicate): normal control (control) group, basal feed with 0 cfu/g *Bacillus* strains;

treatment 1 (T-1) group, basal feed with 1.0×10^5 cfu/g *Bacillus* strains; treatment 2 (T-2) group, basal feed with 1.0×10^6 cfu/g *Bacillus* strains; treatment 3 (T-3) group, basal feed with 1.0×10^7 cfu/g *Bacillus* strains; and the experimental duration was 8 weeks.

The formula of basic rations is listed in Table S2 and the nutrient level was calculated in accordance with "Nutrition of the Rabbit" of the 2th edition in 2010 (Blas and Wiseman 2010). The rex rabbits in all cages had ad libitum access to feed and water without antibiotics throughout the experiments. The body weight and feed intake were recorded every week. All animal experiments were performed according to the Sichuan Agricultural University Animal Care and Use Committee (approval number: SYXKchuan 2014-187).

Sample collection

At the end of both experiments, the blood samples were collected from the heart for serum and then preserved at − 20 °C. Ten rex rabbits (five for each gender) were randomly selected and euthanized for sample collection. The thymus, sacculus rotundus, and vermiform appendix in both experiments were removed and weighed. Subsequently, the contents of the small intestines (duodenum, jejunum, and ileum) and cecum were collected, immediately transferred into liquid nitrogen, and then stored at − 80 °C in both experiments. Fresh tissue samples from the middle part of the jejunum were collected, washed with normal saline, and fixed with 4% paraformaldehyde solution in experiment 2.

Determination of biochemical parameters and antioxidant indexes in serum

Biochemical parameters including urea nitrogen (BUN), total protein (TP), and glucose (GLU) were determined using a GS200 Automatic Biochemical Analyzer (Shenzhen Genius Electronics Co., Ltd., Shenzhen, China). Antioxidant indexes in the serum were determined using commercial reagent kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) following manufacturer's instructions. The antioxidant indexes included superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT), and malonaldehyde (MDA).

Bacterial enumeration in the intestinal contents

The counts of *Lactobacillus* spp., *Bacillus* spp., and *Escherichia coli* were determined by selective media: de Man, Rogosa & Sharpe (MRS), NA, and MacConkey agar. A total of 0.5 g of intestinal content was diluted, spread in the plate of each selective media, and incubated at 37 °C. The procedures of incubation conditions and colony identification were in consistent with those described by Giannenas et al. (2012). The microbial counts were log transformed before statistical analysis.

Quantification of cecal microbiota

Microbial genome DNA of cecal content in experiment 2 was extracted using the E.Z.N.A.™ stool DNA kit (Omega Bio-Tek, Doraville, USA) following the introduction of the manufacturer. The abundance of *Ruminococcus albus*, *Ruminococcus flavefaciens*, *Fibrobacter succinogenes*, and *Prevotella prophyromonas* were determined by quantitative PCR (qPCR) using a CFX96 Connect™ Real-Time system (Bio-Rad, Hercules, USA) and SYBR® Premix Ex Taq™ II (TaKaRa, Dalian, China), and the primers are shown in Table S3. The reaction mixture and cycling protocols used in the present study and the building of standard curves were in accordance with Sun et al. (2016). Copy numbers of microbiota were calculated through the standard curves.

Determination of VFA in cecal contents

The concentrations of VFAs (acetic acid, propionic acid, and butyric acid) in cecal contents were measured using CP-3800 gas chromatograph (Varian Inc., Palo Alto, USA) following the instructions of the manufacturer.

Determination of digestive enzyme activity in the intestinal contents

The samples of duodenum, jejunum, ileum, and cecum contents were weighed and homogenized with saline. The supernatants were collected by centrifugation and assayed for the activities of protease, amylase, and cellulase (CMC-ase, FP-ase) according to the methods in "Isolation, screening, and identification of *Bacillus* strains" section.

Jejunal histomorphology

The tissue sample of jejunum in experiment 2 was stained with hematoxylin and eosin (H&E) using standard paraffin embedding procedures described by Wang et al. (2017). The villus height and crypt depth were measured using Image-Pro Plus 6.1 (Media Cybernetics Inc., Rockville, USA).

Statistical analysis

All data were expressed as mean ± standard deviation (SD). Differences between various treatments were analyzed by one-way analysis of variance (ANOVA) and Duncan's multiple comparison tests using the SPSS 19.0 statistical package (SPSS Inc., Chicago, USA). The level of significance set was $P < 0.05$. The figures were generated by using GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, USA).

Results

Isolation, screening, and identification of *Bacillus* spp.

A total of 122 *Bacillus* spp. were isolated from the rex rabbits. Of these, 109, 100, 30 *Bacillus* strains showed the ability to degrade cellulose, protein, and starch, respectively (data not shown). According to H/C, 19/31/16 isolates out of 109/100/30 showed a higher REA value (REA ≥ 1.90/1.97/1.23) for cellulose/protease/amylase production (Tables S4, S5, and S6). These isolates exhibiting higher REA values were selected for further enzyme activity determination. The highest cellulase (CMC-ase and FP-ase)/protease/amylase activity was exhibited by BSWJ2017120/BSWJ2017091/BSWJ2017001 followed by BSWJ2017023 and BSWJ2017002/BSWJ2017003 and BSWJ2017056/BSWJ2017015 and BSWJ2017004, respectively (Tables S4, S5, and S6). The optimal combination of strains for cellulase (CMC-ase and FP-ase) production was BSWJ2017002, BSWJ2017003, and BSWJ2017001 (Table S7). Additionally, the optimal combination also exhibited better protease activity and amylase activity (Table S7). Based on the above results, three isolates, BSWJ2017002, BSWJ2017003, and BSWJ2017001, were selected for further studies. Biochemical identification and 16S rRNA sequence analysis identified strain BSWJ2017002 as *Bacillus pumilus*, BSWJ2017003 as *Bacillus subtilis*, and BSWJ2017001 as *Bacillus subtilis*. The 16S rRNA sequences of three *Bacillus* strains were submitted to Genebank and the accession numbers were KY996496-KY996498. The three strains were deposited in the China Center for Type Culture Collection (Wuhan, China) (registration numbers: CCTCC AB 2017130, CCTCC AB 2017175, and CCTCC M 2017290).

Growth performance and organ indexes

The effect of three *Bacillus* strains on growth performance of rex rabbits in both experiments is summarized in Table 1. The results showed that average daily feed intake (ADFI) was reduced in rex rabbits supplemented with *Bacillus* strains compared to the control in both experiments. There was no obvious difference ($P > 0.05$) in average daily gain (ADG) between the treatment and control groups in both experiments 1 and 2. The value of feed conversion ratio (FCR) in MC, T-2, and T-3 groups was significantly lower ($P < 0.05$) than that of control group.

The values of thymus, sacculus rotundus, and vermiform appendix indexes in both experiments are presented in Table 1. The thymus index of MC group in experiment 1 and T-1 group in experiment 2 was significantly higher ($P < 0.05$) than that in the control group. However, no significant difference ($P > 0.05$) on the sacculus rotundus index was

Table 1 Effect of *Bacillus* strain dietary supplementation on growth performance and organ indexes of rex rabbits

Parameters	NC/control	LC/T-1	MC/T-2	HC/T-3
Experiment 1				
Weeks 1–4				
ADFI, g/day	122	108	106	102
ADG, g/day	14.56 ± 2.89ab	13.40 ± 4.01b	19.38 ± 3.13a	13.57 ± 2.24b
FCR, g/g	8.69 ± 2.09a	8.61 ± 2.50a	5.57 ± 0.80b	7.68 ± 1.35ab
TI	1.92 ± 0.28a	2.31 ± 0.33a	3.45 ± 0.68b	2.41 ± 0.35a
SRI	1.52 ± 0.38	1.47 ± 0.03	1.66 ± 0.20	1.55 ± 0.29
VAI	4.04 ± 0.27a	4.59 ± 0.70ab	5.21 ± 0.69b	4.24 ± 0.45ab
Experiment 2				
Weeks 1–8				
ADFI, g/day	143.15	137.62	130.75	131.37
ADG, g/day	27.01 ± 1.50	28.06 ± 2.61	27.08 ± 2.29	27.08 ± 1.14
FCR	5.32 ± 0.30a	4.94 ± 0.47ab	4.86 ± 0.42b	4.86 ± 0.21b
TI	2.27 ± 0.25a	3.05 ± 0.66b	2.29 ± 0.24a	2.48 ± 0.28ab
SRI	1.89 ± 0.09	2.07 ± 0.22	2.12 ± 0.19	2.00 ± 0.36
VAI	4.58 ± 0.71a	4.65 ± 0.31ab	5.32 ± 0.28b	4.33 ± 0.38a

Data were expressed as mean ± SD. Means with the different lowercase letters at the same row are significantly different ($P < 0.05$). NC/control group = basal diet with no antibiotics; LC/T-1 group = basal diet containing 1.0×10^5 cfu/g *Bacillus* strains; MC/T-2 group = basal diet containing 1.0×10^6 cfu/g *Bacillus* strains; HC/T-3 group = basal diet containing 1.0×10^7 cfu/g *Bacillus* strains. The ratio of three *Bacillus* strain cells in each combination was 1:1:1

ADFI average daily feed intake, *ADG* average daily gain, *FCR* feed conversion ratio (ADFI/ADG), *TI* thymus index, *SRI* sacculus rotundus index, *VAI* vermiform appendix index

observed among all groups in both experiments. On the other hand, the vermiform appendix index was significantly increased ($P < 0.05$) by 10^6 cfu/g *Bacillus* strains in both experiments.

Biochemical parameters

The biochemical parameters in serum are shown in Fig. 1. For experiment 1, the level of BUN was slightly decreased ($P > 0.05$) in *Bacillus* strain groups as compared to NC, whereas significantly lower BUN level ($P < 0.05$) was observed in T-2 and T-3 group compared with control group in the experiment 2. In experiments 1 and 2, rex rabbits fed with 10^6 and 10^7 cfu/g *Bacillus* strains showed higher TP level ($P < 0.05$) than the controls. Furthermore, the feeding of *Bacillus* strains significantly increased the level of GLU ($P < 0.05$) in experiment 1, while no significant difference was observed ($P > 0.05$) among all groups in experiment 2.

Antioxidant indexes

The antioxidant indexes of serum in both experiments are presented in Fig. 2. The activities of SOD, GSH-Px, and CAT in both experiments were found to be improved in all rex rabbits fed with *Bacillus* strains compared to the controls. For experiment 1, the SOD activity was significantly increased ($P < 0.05$) in MC group, but there was no significant difference ($P > 0.05$) between the *Bacillus* group and control group in GSH-Px and CAT activities. For experiment 2, the activities of SOD and GSH-Px were found to be significantly elevated ($P < 0.05$) in rex rabbits provided by *Bacillus* strain diets. However, only T-3 group exhibited significantly higher CAT activity ($P < 0.05$) than that in control group. On the other hand, LC group in experiment 1 and T-2 group in experiment 2 showed lower MDA contents ($P < 0.05$) compared to the controls.

Bacterial counts in cecal contents

The counts of *Lactobacillus* spp., *Bacillus* spp., and *Escherichia coli* in cecal contents are presented in Fig. 3. For experiment 1, *Bacillus* strain treatment resulted in a remarkable increase ($P < 0.05$) of *Lactobacillus* spp. counts in the cecal contents compared with NC group. Moreover, obvious increase in *Bacillus* spp. counts in cecal contents was found ($P < 0.05$) in both MC and HC groups compared with NC group, whereas the significant decrease in *Escherichia coli* counts in cecum was only revealed ($P < 0.05$) in HC group. In experiment 2, the inclusion of *Bacillus* strains in the diet significantly enhanced ($P < 0.05$) the *Lactobacillus* spp. and *Bacillus* spp. counts in the cecal contents in comparison with control. The count of *Escherichia coli* decreased significantly ($P < 0.05$) in experimental groups.

Microbial populations quantified by q-PCR in cecal contents

The abundance of microbiota in cecal contents is shown in Fig. 4. In experiment 2, the abundance of *Ruminococcus albus* of cecal contents in the groups fed with *Bacillus* strains was slightly elevated ($P > 0.05$) in relation to control group, whereas the abundance of *Ruminococcus flavefaciens* and *Fibrobacter succinogenes* was significantly increased

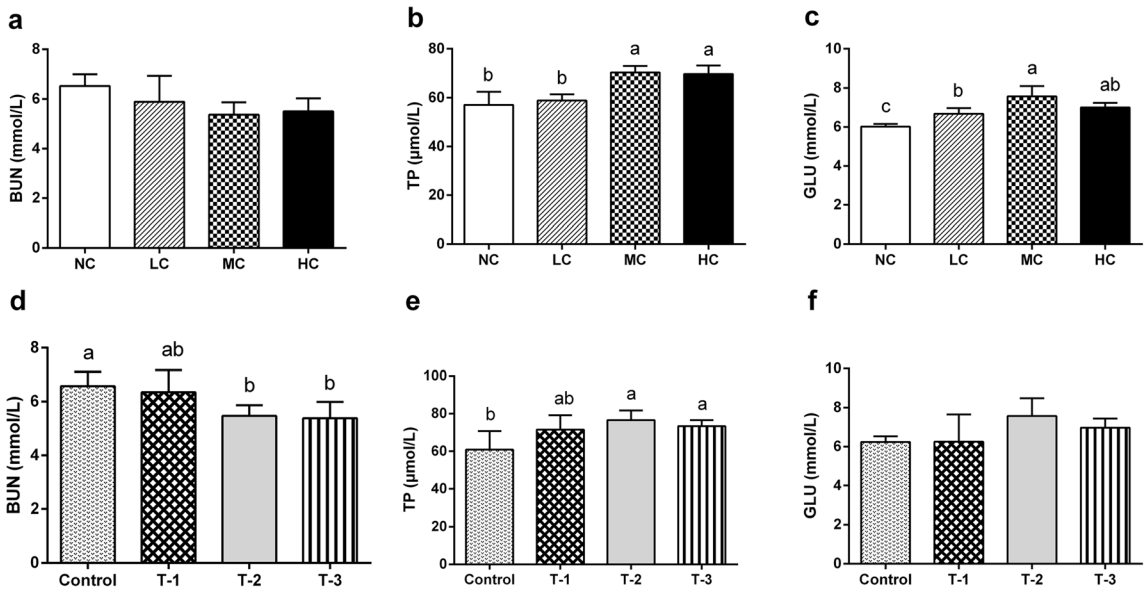

Fig. 1 Effects of *Bacillus* strain dietary supplementation on biochemical parameters in serum of rex rabbits. Data were expressed as mean ± SD. Means with the different lowercase letters at the same row are significantly different ($P < 0.05$). **a–c** The contents of BUN, TP, and GLU in experiment 1. **d–f** The contents of BUN, TP, and GLU in experiment 2. BUN, urea nitrogen; TP, total protein; GLU, glucose

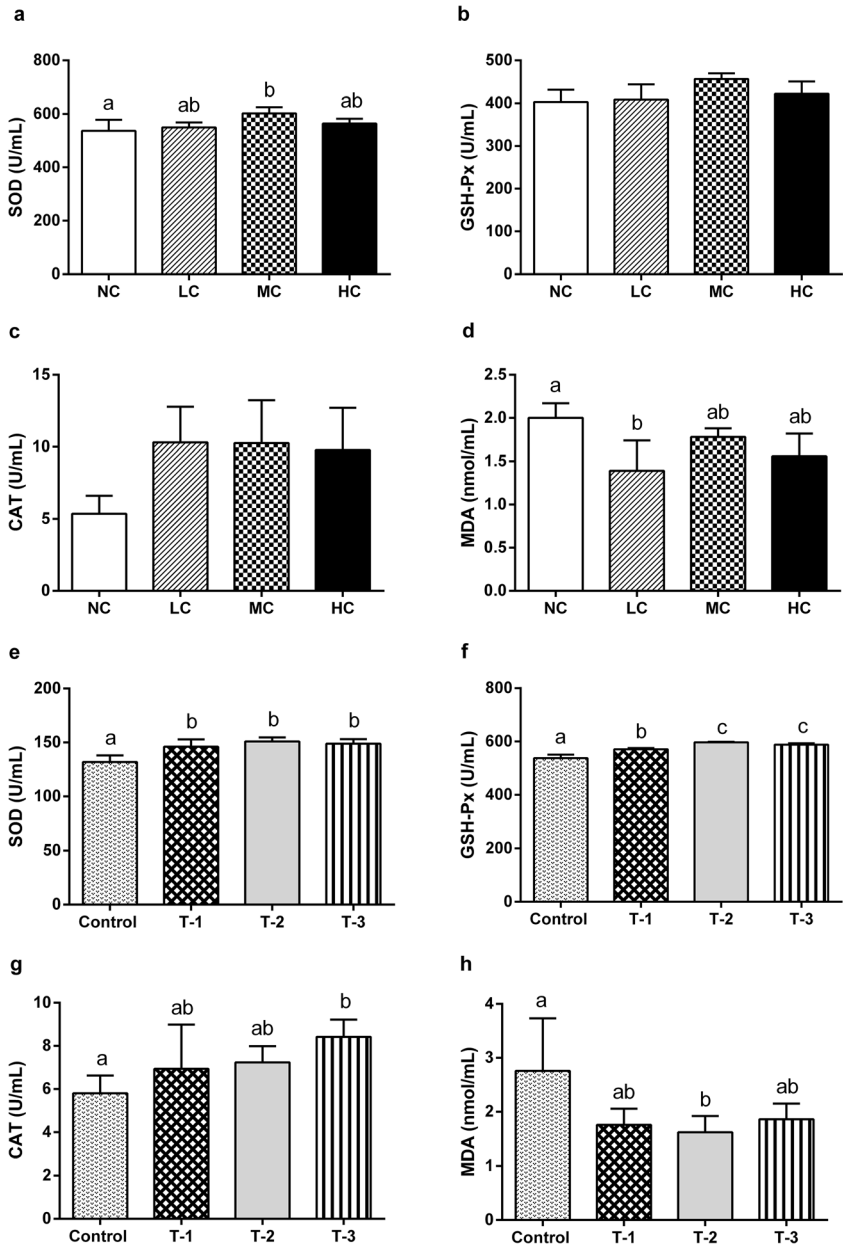

Fig. 2 Effect of *Bacillus* strain dietary supplementation on antioxidant indexes in serum of rex rabbits. Data were expressed as mean ± SD. Means with the different lowercase letters at the same row are significantly different ($P < 0.05$). **a–c** SOD activity, GSH-Px activity, CAT activity, and MDA content in experiment 1. **d–f** SOD activity, GSH-Px activity, CAT activity, and MDA content in experiment 2. SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; CAT, catalase; MDA, malonaldehyde

($P < 0.05$) after 8 weeks of *Bacillus* strain feeding. Moreover, the abundance of *Prevotella prophyromonas* in T-2 group was higher ($P < 0.05$) than control group.

VFA in cecal contents

As shown in Fig. 5, the supplementation of *Bacillus* strains increased the VFA concentrations of cecal contents in both experiments. For experiment 1, the concentration of acetic acid in cecal contents was significantly elevated ($P < 0.05$) in LC and MC group compared with NC group, whereas the propionic acid concentration of all the *Bacillus* groups showed no significant difference ($P > 0.05$). Besides, the significant increase ($P < 0.05$) of butyrate acid concentration in the cecum was only revealed in MC group. For experiment 2, rex rabbits fed with *Bacillus* strains had greater ($P < 0.05$) concentrations of VFAs (acetic acid, propionic acid, and butyric acid) in cecal contents than those in control group.

Digestive enzyme activities in the intestinal contents

The digestive enzyme activities in intestinal contents in two experiments are presented in Fig. 6. The digestive enzyme activities were higher in all *Bacillus* strains group than the control. For experiment 1, in MC and HC groups, both protease and amylase activities of duodenum were significantly increased ($P < 0.05$) compared to NC group. Similarly, MC and HC groups also exhibited significantly increased cellulase

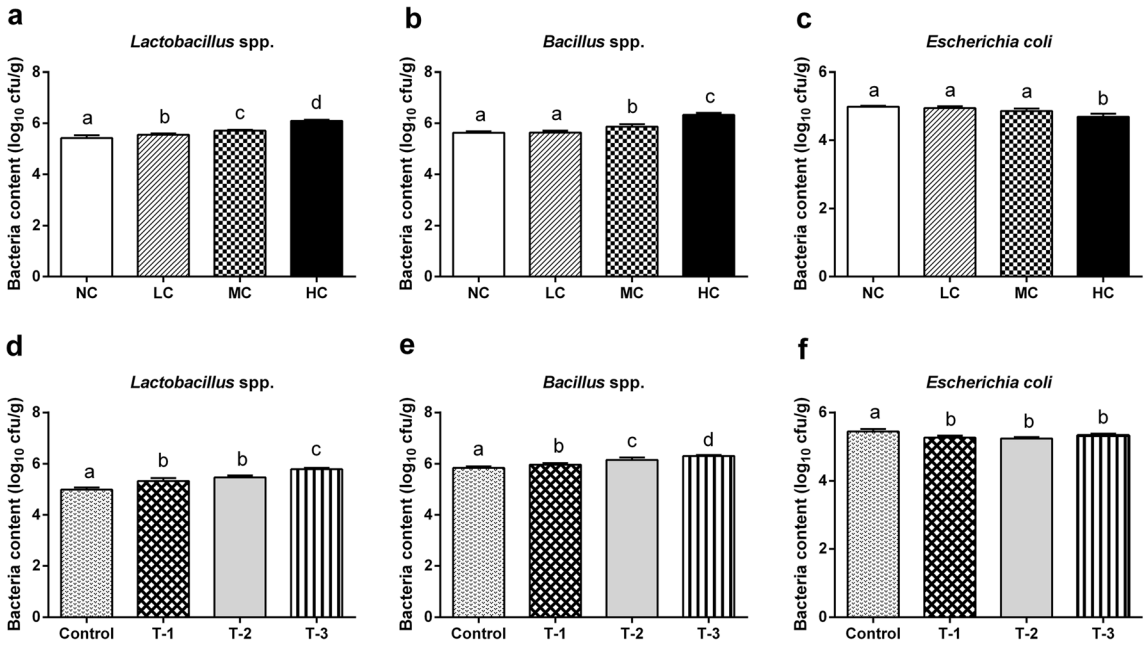

Fig. 3 Effect of *Bacillus* strain dietary supplementation on the bacterial counts in the cecal contents of rex rabbits. Data were expressed as mean ± SD. Means with the different lowercase letters at the same row are significantly different ($P < 0.05$). **a–c** The counts of *Lactobacillus* spp., *Bacillus* spp., and *Escherichia coli* in experiment 1. **d–f** The counts of *Lactobacillus* spp., *Bacillus* spp., and *Escherichia coli* in experiment 2

(CMC-ase and FP-ase) activity ($P < 0.05$) in the cecum. Significantly higher protease activity of jejunum was detected ($P < 0.05$) in LC and MC group compared to control group. Furthermore, the amylase activity of jejunum in MC group was significantly elevated ($P < 0.05$) in relation to NC group. Significant differences in the protease and amylase activities of ileum were observed in *Bacillus* strain groups compared with control group.

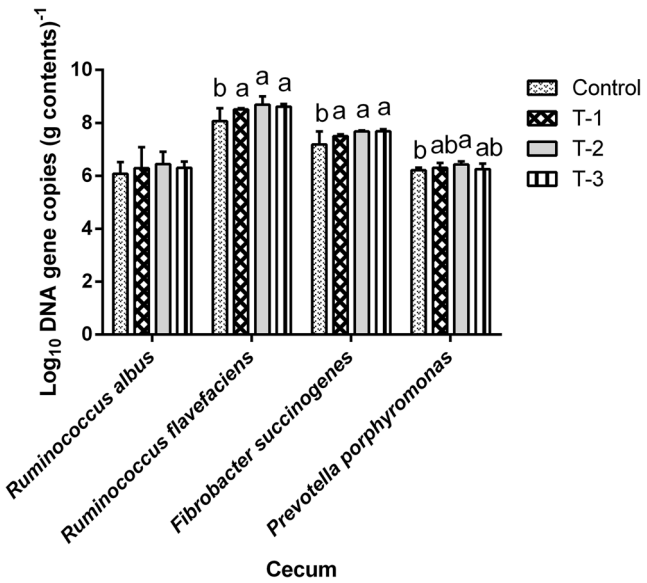

Fig. 4 Effects of *Bacillus* strain dietary supplementation on microbial populations in the cecal contents of weaning rex rabbits in experiment 2. Data were expressed as mean ± SD. Means with the different lowercase letters at the same row are significantly different ($P < 0.05$)

For experiment 2, the protease activity of duodenum in three treated groups and amylase activity of duodenum in T-2 group were significantly elevated ($P < 0.05$) after 8 weeks of *Bacillus* feeding. Meanwhile, T-2 and T-3 groups exhibited significantly increased protease and amylase activities ($P < 0.05$) in the jejunum. Moreover, the increase in protease activity of ileum was observed ($P < 0.05$) in both T-2 and T-3 groups compared with control group, whereas the significant increase in amylase activity of ileum was only revealed ($P < 0.05$) in T-3 group. The CMC-ase activity of cecum was found significantly elevated ($P < 0.05$) in T-2 treatment compared to controls. The FP-ase activity of cecum also showed significant difference ($P < 0.05$) in three experimental groups compared with control group.

Jejunal morphology

The results describing the effect of three *Bacillus* strains on jejunal morphology of rex rabbits in experiment 2 are presented in Fig. 7. In experiment 2, rex rabbits fed with *Bacillus* strains diet had greater villus height and villus height/crypt depth ($P < 0.05$) in jejunum. Significantly shallower crypt depth of jejunum was also observed ($P < 0.05$).

Discussion

The beneficial effects of probiotic *Bacillus* on growth and digestion have not been widely explored (Hu et al. 2018; Guo et al. 2017). The present study reported mixture of three

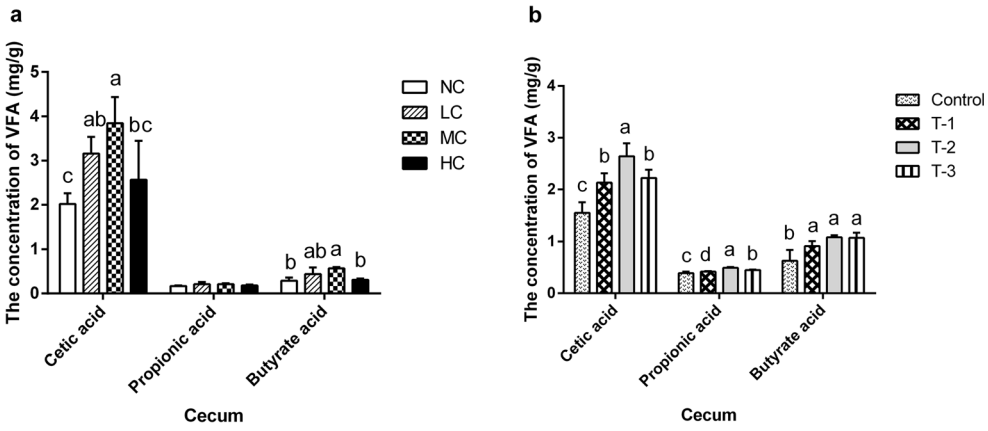

Fig. 5 Effects of *Bacillus* strain dietary supplementation on VFA in the cecal contents of rex rabbits. Data were expressed as mean ± SD. Means with the different lowercase letters at the same row are significantly different ($P < 0.05$). **a** The concentrations of cetic acid, propionic acid, and butyrate acid in experiment 1. **b** The concentrations of cetic acid, propionic acid, and butyrate acid in experiment 2

Bacillus strain with high performance on cellulase, protease, and amylase activities, which could improve the growth performance of young and weaning rex rabbits by enhancing digestive function and anti-disease ability.

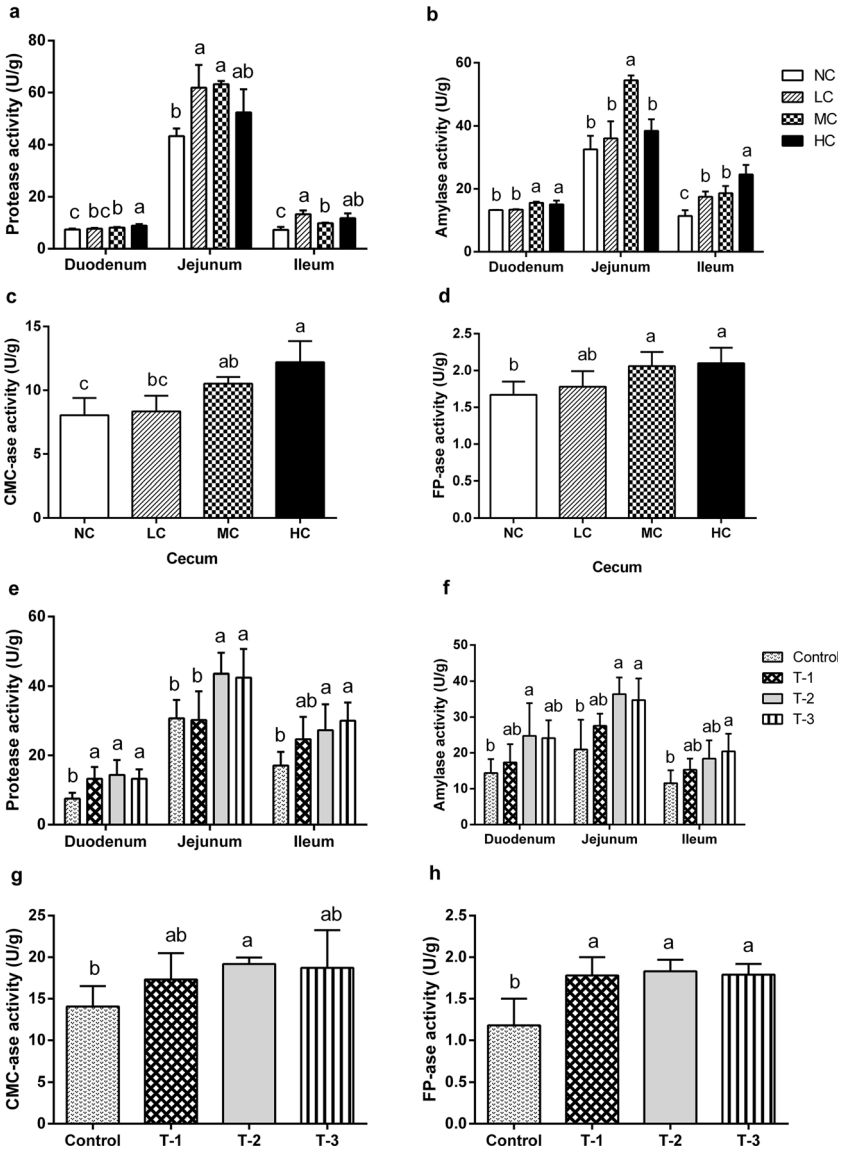

Fig. 6 Effects of *Bacillus* strain dietary supplementation on digestive enzyme activities in the intestinal contents of rex rabbits. Data were expressed as mean ± SD. Means with the different lowercase letters at the same row are significantly different ($P < 0.05$). **a**, **b** Protease and amylase activities of duodenum, jejunum, and ileum in experiment 1. **c**, **d** CMC-ase and FP-ase activities of cecum in experiment 1. **e**, **f** Protease and amylase activities of duodenum, jejunum, and ileum in experiment 2. **g**, **h** CMC-ase and FP-ase activities of cecum in experiment 2. FP-ase, filter paper-ase; CMC-ase, carboxymethyl cellulase

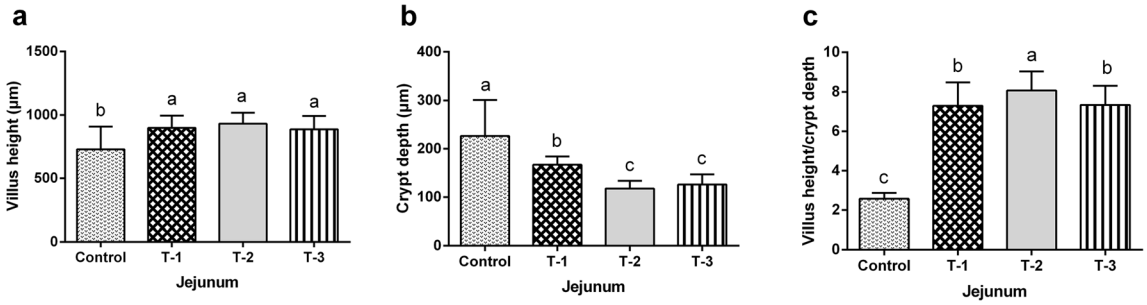

Fig. 7 Effects of *Bacillus* strain dietary supplementation on jejunal morphology of weaning rex rabbits in experiment 2. Data were expressed as mean ± SD. Bars with different letters are significantly different ($P < 0.05$). **a–c** Villus height, crypt depth, and villus height/crypt depth ratio of jejunum in experiment 2

Our research showed that 10^6 cfu/g *Bacillus* strains significantly decreased the FCR and increased the vermiform appendix index of both young and weaning rex rabbits. Similarly, the growth performance and immune organ indexes of Cherry Valley ducks were also reported to be enhanced by feeding with 10^6 cfu/g *Bacillus* subtilis (Guo et al. 2016). Improved growth and feed efficiency were related to serum biochemical indexes and intestinal morphology (Dawood et al. 2019). Serum biochemical indexes could reflect the status of nutrition and health of the body. In this study, *Bacillus* strains increased TP and GLU contents and decreased BUN contents in both of the experiments, suggesting that the addition of *Bacillus* strains promoted the metabolism of protein and carbohydrates (Oh et al. 2008; Stanley et al. 2002). Villus height, crypt depth, and the ratio are important indicators to reflect intestinal morphology. The increase of villus height increased the contact area between intestinal tract and chyme, thus enhanced the digestion and absorption of nutrients. It also increased the swing to make it more difficult for pathogens to colonize in the intestines (Caspary 1992). The shallower crypt depth indicated that the maturation rate of intestinal epithelial cell was raised and the secretory function and chemical digestion were enhanced. Meanwhile, it also showed that the growth of intestinal mucosal epithelial cells was accelerated and the ability to repair intestinal injury was heightened (Hampson 1986). In this study, a markedly raising jejunal morphology was observed in weaning rex rabbits fed with *Bacillus*.

Antioxidant enzymes which mainly included SOD, GSH-Px, and CAT could protect the body from oxidative damages and also enhance the defense and immune ability of the body (Rudneva 1997; Esposito et al. 2000). MDA can connect with albumin to form adduct antigen, which is then swallowed by macrophages to cause cell damages, resulting in physiological disorders and diseases (Niki 2010). Gong et al. (2018) found that the addition of *Bacillus* in diet could increase the activities of SOD and T-AOC as well as reduce MDA level of broilers. Our results in two experiments revealed that the MDA content and activities of SOD, GSH-Px, and CAT were positively affected by *Bacillus*, indicating that the administration of *Bacillus* strains could strengthen anti-disease ability by improving the antioxidant capacity.

VFAs can reduce the pH in gastrointestinal tract and promote the proliferation and activation of immune cells. Therefore, they are capable to promote the digestion and absorption of nutrients, improve immunity, and thus reduce the occurrence of intestinal diseases (Maria et al. 2002; Wierdsma et al. 2009). In the present study, *Bacillus* strains at a middle dose (1.0×10^6 cfu/g) in young rex rabbits and three added doses in weaning rex rabbits obviously elevated the concentrations of acetic acid, propionic acid, and butyric acid in the cecum. Correspondingly, these increases led to reduction in cecal *Escherichia coli* count. It is known that VFA production is connected with carbohydrate fermentation by cellulolytic bacteria and amylolytic bacteria (Van Soest 1993). *Lactobacillus* spp. and *Bacillus* spp. could enhance the activity of digestive enzymes to raise carbohydrate digestion (Dawood et al. 2019; Ten et al. 2004; Hu et al. 2018). Previous researches also indicated that they could induce non-specific immune response by activating monocytes and natural killer cells and induce specific immune response by promoting the production of Th1 cytokines IL-2, IFN-γ, and TNF-α, which consequently eliminated invasive pathogenic microorganisms (Pagnini et al. 2009; Castillo et al. 2011). *Ruminococcus albus*, *Ruminococcus flavefaciens*, and *Fibrobacter succinogenes* are the main cellulolytic bacteria to decompose cellulose into volatile fatty acids (Michalet-Doreau et al. 2001). *Prevotella prophyromonas* is sugar fermentation microorganism and can digest plant non-fiber polysaccharides, starch, xylose, and pectin (Kopečný et al. 2003). Consistent with VFA results, the counts of *Lactobacillus* spp. and *Bacillus* spp. in the cecum of young and weaning rex rabbits were raised in experimental groups when compared to control group. The abundances of *Ruminococcus flavefaciens*, *Fibrobacter succinogenes*, and *Prevotella prophyromonas* in the cecum of weaning rex rabbits were also increased. These findings indicated that the improvement of digestive function and anti-disease ability of *Bacillus* strains may be related to the improvement of VFA concentration and intestinal microbiota.

In current study, we found that the cellulase (CMC-ase and FP-ase) activity in the cecum as well as the protease and amylase activities in small intestines of young and weaning rex rabbits were enhanced after *Bacillus* strain administration. The results indicated that digestive function was heightened by *Bacillus* strains. Cellulase is mainly produced by cellulolytic microorganisms and is composed of endo-β-1,4-glucanases, exo-β-1,4-glucanases, and β-glucosidases (Gidenne and Licois 2005; Béguin and Aubert 1994). The degradation of fiber is completed under the synergistic action of three enzymes, and changes of the activity of any enzyme could affect its degradation. CMC-ase activity reflects the activity of endo-β-1,4-glucanases and FP-ase activity represents the total cellulase activity (McCleary et al. 2017). Protein and starch are mostly digested by protease and amylase produced by mammals (minor digested by intestinal bacteria) (Switzar et al. 2013; Drozdowski and Thomson 2006). The results of in vitro studies also indicated that the mixture of three *Bacillus* strain showed high cellulose, protease, and amylase activities. Therefore, we assumed that the rise of cellulase activity might be due to the proliferation of cellulolytic microorganisms including *Bacillus*. Meanwhile, the augmentation of protease and amylase activities was probably because *Bacillus* strains stimulated the production of endogenous enzymes in rex rabbits. The contribution rate of digestive enzymes secreted by *Bacillus* strains needs to be further studied. Activated *Bacillus* could germinate into vegetative cells in the intestinal tract, then release metabolites and regulate intestinal microbiota, while inactivated *Bacillus* could not proliferate and produce beneficial substances such as digestive enzymes (Guo et al. 2017). Our results showed that the intestinal microbiota and digestive enzymes changed significantly. For these reasons, we speculated that inactivated strains had no obvious impact on growth performance, digestive function, and anti-disease ability.

In conclusion, our results indicated that supplementation with *Bacillus* strains at 1.0×10^6 cfu/g could promote the growth performance of young and weaning rex rabbits by enhancing digestive function and anti-disease ability, which were associated with the positive influence of *Bacillus* strains on immune organ indexes, serum biochemical parameters, antioxidant capacity, gut microbiota, VFA concentrations, intestinal morphology, and intestinal digestive enzyme. Further studies are needed for exploring the feasibility of its commercial application in rabbits.

Authors' contributions JW, DZ, XN, BW, and HL designed the study. JW, BW, and HL performed the experiments. JW, KP, BJ, YZ, PW, WZ, AK, ZY, and YZ analyzed the data. JW and DZ wrote the manuscript. JW, WZ, and LL revised the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All experimental procedures were performed according to the Animal Care and Use Committee of Sichuan Agricultural University (approval number: SYXKchuan 2014-187).

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