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Dietary supplementation with *Bacillus* can improve the growth and survival of the kuruma shrimp *Marsupenaeus japonicus* in high-temperature environments

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Abstract This study examined the effects of a commercial *Bacillus* complex used as probiotics on the performance of *Marsupenaeus japonicus* under high water temperature after 60 days of rearing. Growth and survival rate of *M. japonicus* were significantly increased in the probiotics-treated group ($P < 0.05$). *Vibrio* count and percentage of *Vibrio* in the intestinal tracts of *M. japonicus* reared with *Bacillus* were significantly lower than in other treatments ($P < 0.05$). The expressions of immune-related gene including prophenoloxidase, lysozyme, cytosolic manganese superoxide dismutase and hemocyanin subunit L in *M. japonicus* were significantly increased in the *Bacillus*-treated group ($P < 0.05$). The results indicated that *Bacillus* can be used as a diet supplement to minimize the damages caused by free radicals generated from insufficient oxygen metabolism due to the high-temperature stress, and to enhance immunity and activate the immune response levels. Our findings can improve the growth and survival rates of *M. japonicus* during the high-temperature farming period.

Keywords *Bacillus* · *Marsupenaeus japonicus* · Growth · Intestinal bacteria · Immune status · High temperature

Introduction

Marsupenaeus japonicus (Bate, 1888) is an important commercial species cultured in the coastal areas of southeast China. The slow growth and high mortality are the major obstacles hindering the development of *M. japonicus* industry. During high-temperature periods, the survival rate of *M. japonicus* is very low and growth is normally inhibited (You et al. 2010). *Vibrio* and viral infections are usually serious and can cause huge economic losses (Hewitt and Duncan 2001; Greg et al. 2002). Therefore, enhancing the

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shrimps' immunity, suppressing pathogenic infections and accelerating the shrimps' growth rate are critical in *M. japonicus* farming during the summer.

Previous studies have demonstrated that high temperature could affect the metabolism and immunity of shrimps. Excessive oxygen consumption will lead to excessive reactive oxygen species (ROS), which inflict oxidative damage to organism (Fridovich 1995; Lopez-Martinez et al. 2008; Cui et al. 2011). Long-term high-temperature stress can reduce the survival rate of shrimps through deteriorating their immunity (Hewitt and Duncan 2001). Expression levels of several genes related to stress and immune response can serve as important indicators for organic high-temperature stress and immunity. For example, hemocyanins are related to oxygen supply and immunity of organism (Destoumieux-Garzon et al. 2001; Adachi et al. 2003). ProPO and SOD are important components of organic antioxidant defense systems (Campa-Córdova et al. 2002; Wang and Zhang 2008), and the Lys expression level is reflective of the defense capacity against pathogenic microorganism invasion (Burge et al. 2007).

Probiotic application in aquaculture is a significant accomplishment within the field of aquatic science and technology. Probiotics such as *Bacillus* can regulate water quality, suppress pathogenic microorganisms, provide digestive enzymes, activate growth factors and enhance organic immunity (Moriarty 1998; Verschuere et al. 2000; Farzanfar 2006; Keysami et al. 2007; Decamp et al. 2008; Tseng et al. 2009; Liu et al. 2010). Using probiotics to suppress the multiplication of pathogenic bacteria is a significant shrimp farming technique that has been widely used in China. Probiotics are generally applied by water sprinkling, which can effectively regulate water quality and improve the growth and survival rate of shrimp (Ziaei-Nejad et al. 2006; Liu et al. 2009; Zhou et al. 2009). Adding probiotics as a dietary supplementation is an alternative way, which can optimize the microflora in shrimp intestines, enhance growth and improve immunity of shrimp.

The aim of this study was to understand the reactive mechanisms of shrimps to high-temperature stress and to determine the effectiveness of *Bacillus* application in shrimp farming during high-temperature periods. In this study, a method for adding a *Bacillus* composite to feedstuff is presented. The effectiveness of probiotics in mitigating high-temperature stress and improving the growth and survival rates of shrimp was evaluated. The bacteriological analysis of intestinal tracts was investigated, and the antagonistic effect of feeding compound *Bacillus* on shrimp immunity during a high-temperature period was also determined.

Materials and methods

Shrimps and probiotics

Marsupenaeus japonicus juveniles from the same spawners, averaging 1.04 ± 0.21 g in body weight and 4.32 ± 0.26 cm in body length, were obtained from the experimental base of Xiamen University in Fujian, China, in June 2010. The spawners used for breeding were caught from Xiapu (Fujian, China) and transported to a breeding farm at the experimental base in April 2010. Spawning was induced through eyestalk ablation, and the post-larval shrimp were bred in indoor cement ponds with sizes suitable for the experiments. A total of 2,400 shrimps were acclimatized in stocking tanks for 7 days before processing and then randomly assigned to groups.

The probiotic used in this study was a commercial *Bacillus* complex (LISHENGSU, South China Sea Fisheries Institute, Chinese Academy of Fishery Science, China)

containing two species of *Bacillus* (*Bacillus subtilis* and *Bacillus licheniformis*) with a count of $1.03 \pm 0.36 \times 10^{10}$ CFU/g.

Diet preparation

A pretreated commercial manufactured feed for shrimps (LUCKY STAR, Hung Kuo Industrial Co., LTD, Taiwan, China) was used as a dietary source in this study. For probiotic treatment group, the feed was covered with 10 g/kg probiotic using 2.0 % fish oil as the carrier, with a final dose of $1.0 \pm 0.2 \times 10^8$ CFU of *Bacillus* per gram of diet. The control diet was top-coated with 2.0 % fish oil but lacked probiotic (Tseng et al. 2009). Diets were dried in a drying cabinet using an air blower at 37 °C for 2 h and stored at 4 °C until used. The bacterial count of the diet was examined every week using the spread plate method to verify the concentration of the probiotic, and to check for possible contamination.

Experimental design

The experiment was conducted in eight indoor $2 \times 2.5 \times 1.2$ m (length × width × height) cement ponds. The water levels of each pond were maintained at 0.9 m deep through the experiment. For each pond, three-fourths of the surface area was covered with black film to maintain water transparency in the 30–40 cm range and to avoid direct light exposure during the feeding time in the evening. The experimental subjects were divided into a treatment group (with *Bacillus*) and a control group (without *Bacillus*). Each group consisted of four replicates, and each replicate contained 300 shrimps.

Daily management

Shrimps were fed twice a day (0800 and 1900 in a proportion of 3:7) at a wet weight of 5–8 % for 60 days. The waste was collected every morning by siphoning the ponds, and 20 % of the water was changed every 2 days. Natural photoperiod was used in this study, and the water was aerated continuously. Seawater (salinity 28–34 g/L and pH 8.0–8.5) filtered through sand filter and cotton bag filters was stored in an indoor reservoir. The water temperature was recorded at 10:00 a.m. every day.

Measurement of survival and growth

For growth measurement, 50 shrimps were randomly sampled from each pond for wet weight at the beginning and end of the experiment. After the experiment was completed, all the live shrimps were harvested and counted to obtain the survival rates. The specific growth rates (SGRs) were calculated by the following equation: $\text{SGR} = (\ln W_t - \ln W_0) \times 100/t$, where W_0 and W_t are the initial and final wet weight of the shrimp and t is the sampling interval. The yield of shrimp was calculated by the following equation: Yield of shrimp $= C_t \times W_t$, where C_t was the total number of shrimp at the end of the experiment and W_t was the final wet weight of shrimp.

Bacteriological study of intestines

A number of heterotrophic bacteria and *Vibrio* in the intestinal tracts of shrimps from each group were assayed on days 15, 30 and 60. Five individual shrimps were randomly

collected from each pond. The shrimps were dissected to obtain the complete intestinal tract in a sterile environment. The intestines were weighted and homogenized in a sterilized glass homogenizer with 10 mL of sterilized physiological saline. The homogenate was then serially diluted (tenfold), and 100 μL of the diluted liquid was spread onto a plate. Each sample was bacteriologically analyzed in triplicate. The heterotrophic bacterial count was determined using 2166E agar culture medium. The bacteria were cultivated at 30 °C for 24 h, and the number of colonies was counted. For the *Vibrio* count, samples were plated on thiosulfate citrate bile salt agar (TCBS agar, Oxoid, UK) and incubated at 30 °C for 24 h before the colonies were counted.

Immune status analysis

A pooled sample of six shrimps from each pond was used to conduct total RNA extraction and immune-related gene expression analysis. The hemolymph was collected, and hemocytes were collected immediately according to the methods described by Zhou et al. (2010). RNA was extracted from hemocytes and hepatopancreas using Trizol Reagent (Invitrogen, USA) according to the manufacturer's protocol. The quality of the total RNA was examined by 1.2 % agarose gel electrophoresis and quantified on a NanoDrop 1,000 spectrophotometer (Thermo Scientific, Wilmington, USA).

Reverse transcription (RT) was achieved by using 100 U of SuperScript™ III Reverse Transcriptase (Invitrogen, USA) to transcribe poly $(A)^+$ RNA from total RNA (1 μg) with oligo-$d(T)_{18}$ as the primer in a 25-μL reaction according to the manufacturer's protocol.

mRNA expression levels of the immune-related genes such as proPO, Lys and cytMnSOD in hemocytes and HcL in the hepatopancreas were measured by real-time RT polymerase chain reaction (RT-PCR) on an ABI7300 real-time PCR system (Applied Biosystems Foster City CA, USA) with SYBR green II. The β-actin gene was used as an internal control. The specific primers for the target genes were designed with the aid of Primer Premier 5.0 (PREMIER Biosoft International, Palo Alto, CA, USA) and synthesized by Genscript (Nanjing, China). The sequences and GenBank accession numbers are shown in Table 1.

Amplification reactions were performed in a total volume of 20.0 μL, containing 10.0 μL of 2 × SYBR® Premix Ex Taq™ (TaKaRa Biotechnology (Dalian) Co., Ltd, Dalian, China), 0.8 μL (each) of 10 μM forward and reverse primers, 2.0 μL of the cDNA

Table 1 Immune-related genes of *M. japonicus* and primers used to detect the genes by quantitative real-time PCR

Gene	Primer sequence (5′–3′)	GenBank #	Position	Product (bp)
proPO	F: CCGAGTTTTGTGGAGGTGTT R: GAGAACTCCAGTCCGTGCTC	AB065371	565–695	131
Lys	F: CCTTTCCAGGGAAGACATCA R: TCTGGAAGATGCCGTAGTCC	AB080238	114–259	116
cytMnSOD	F: TCAAGGAGCAATTGCACAAG R: CTTCTTGTGATTGGGGCAGT	GQ181123	522–657	136
HcL	F: GACAGGGAAGGCAAACACAT R: CCAAGCACAATGTGAGCAGT	EF375711	998–1,134	137
β-Actin	F:CAGTCTTCCCCTCCATCGT R: TGCTCGATGGGGTATTTCA	GU645580	89–221	133

ProPO prophenoloxidase, *Lys* lysozyme, *cytMnSOD* cytosolic manganese superoxide dismutase, *HcL* hemocyanin subunit L, *F* forward primer, *R* reverse primer

sample (diluted 1:5 with PCR-grade water), 0.4 μL of ROX and 6.0 μL of PCR-grade water. PCR amplification was conducted with the following program: 95 °C for 10 s, followed by 40 cycles of 95 °C for 10 s and 60 °C for 31 s. Each sample was performed in triplicate, and DEPC water replaced the template in the negative control.

Melting curve analysis of the PCR amplification products was performed at the end of each PCR to establish that only one PCR product was amplified and detected. The results are expressed as the fold change in expression, relative to the β-actin gene, according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Differences in the target gene relative to β-actin gene expression between the control and treatment groups were evaluated to assess changes in gene expression.

Statistical analyses

The data were presented as the mean ± SD. One-way ANOVA was used for statistical analyses of the data with a threshold significance level of $P < 0.05$. Duncan's multiple-range tests (Statistical Package Social Science, SPSS, version 17.0) were used to assess the significance of differences between treatments.

Results

Water temperature

The water temperatures during the rearing experiment are shown in Fig. 1. The water temperature ranged from 30.3 to 33.1 °C during the experiment. Within the experiment period, there were 50 days with temperatures in excess of 31 °C. The water temperature remained within a daily range of <1 °C per day.

Survival and growth

The growth performance data of the shrimp are given in Table 2. After 60 days of breeding, the final weight, SGR, yield and survival rate were significantly higher ($P < 0.05$) in the treatment group than in the control group; on average, these parameters were 43.58, 48.78, 192.64 and 102.96 % higher than in the controls, respectively.

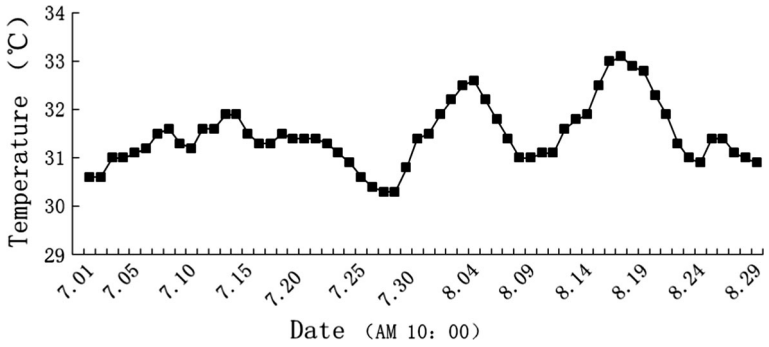

Fig. 1 Variation in the water temperature during the rearing experiment (recorded at 10:00 a.m.). Results are presented as means of the observations from eight ponds

Bacteriological analysis

The intestinal bacteria were counted in the two groups of shrimps, and the results are shown in Table 3. The total heterotrophic bacterial counts in the intestinal tracts of the two groups were gradually reduced, and there was no significant difference between the treatment group and the control group ($P > 0.05$). The intestinal *Vibrio* counts and the percentages of *Vibrio* in the probiotic treatment group were significantly lower than in the control group ($P < 0.05$), followed by a gradually decreasing pattern in the treatment group during the rearing experiment.

Immune status analysis

The mRNA levels of certain immune-related genes were analyzed in the hemocytes and hepatopancreas of the two groups after 60 days of breeding in high-temperature water (Fig. 2). The expression levels of the cytMnSOD, Lys and ProPO genes in hemocytes, and of the HcL gene in hepatopancreas of the *M. japonicus* treated with *Bacillus* were significantly higher than those in the control group (by 18.5-, 3.8-, 9.4- and 8.2-fold, respectively) ($P < 0.05$).

Discussion

Most scholars believed that the suitable water temperature for *M. japonicus* is 18–32 °C, and the optimal temperature for its growth is within the range of 23–30 °C (Liao and Chen 1994; Greg et al. 2002). There were 50 days with temperatures in excess of 31 °C, and the diurnal temperature range of water remained within 1 °C during the experiment, which indicated that the shrimps lived in a high-temperature environment for most of the time during the breeding experiment (Fig. 1).

The growth and survival of shrimps depends on their physiological metabolism status as well as environmental factors (such as temperature, pH, pathogenic bacteria and salinity), nutrient levels and immune function. Significantly reduced shrimp ingestion under high-temperature environments can damage physiological metabolism and weaken immunity, thereby affecting the growth and survival. The previous studies indicated that probiotics as a feed additive could improve the digestive enzyme activities of shrimps, promote host digestion and absorption and improve metabolism and growth (Farzanfar 2006;

Table 2 Growth performance of *M. japonicus* reared with or without *Bacillus* feed supplementation after 60 days of breeding ($n = 4$, mean ± SD)

Items	Group		ANOVA *P*
	Control	Treatment	
Final weight (g)	2.18 ± 0.17^{a}	3.13 ± 0.25^{b}	0.005
SGR (%)	1.23 ± 0.13^{a}	1.83 ± 0.13^{b}	0.005
Yield of shrimp (g)	197.65 ± 7.77^{a}	578.40 ± 24.87^{b}	0.000
Survival rate (%)	30.44 ± 3.50^{a}	61.78 ± 4.76^{b}	0.001

Data in the same line with different superscripts are significantly different ($P < 0.05$)

SGR specific growth rate

Table 3 Heterotrophic bacteria counts and *Vibrio* counts in digestive tracts of *M. japonicus* reared with or without *Bacillus* feed supplementation ($n = 20$, mean ± SD)

Time (days)	Group	Item		
		Vibrio count ($\times 10^5$ CFU g^{-1})	Heterotrophic bacteria count ($\times 10^7$ CFU g^{-1})	Percentage of *Vibrio* (%)
15	Treatment	15.33 ± 4.10^a	11.34 ± 2.44	1.27 ± 0.18^a
	Control	233.16 ± 54.58^b	13.71 ± 1.25	15.08 ± 3.03^b
30	Treatment	9.21 ± 2.60^a	8.88 ± 1.74	1.13 ± 0.22^a
	Control	300.45 ± 55.24^b	9.68 ± 3.71	27.04 ± 3.16^b
60	Treatment	8.76 ± 0.57^a	8.10 ± 0.76	1.06 ± 0.09^a
	Control	339.33 ± 29.11^b	8.72 ± 3.41	29.37 ± 1.26^b

Data with different superscripts indicate significant differences ($P < 0.05$) between two groups at the same time

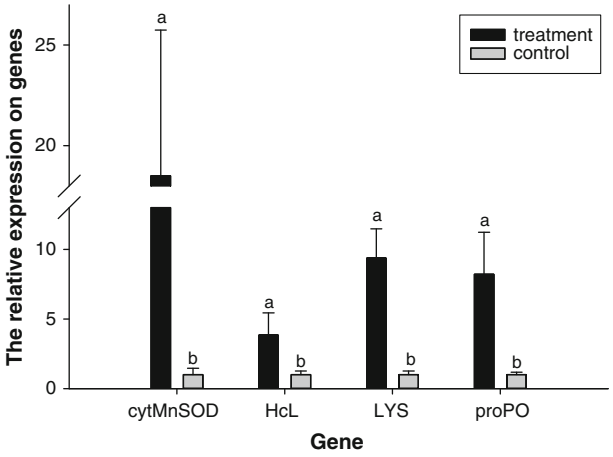

Fig. 2 Analysis of the expression of the immune-related genes (cytMnSOD, Lys and ProPO) in hemocytes and the HcL gene in the hepatopancreas in the treatment and control groups of *M.japonicus* by SYBR Green real-time RT-PCR after 60 days of breeding ($n = 4$, mean ± SD). Six shrimp randomly taken from each pond were pooled in a replicate, and all real-time reactions were performed in triplicate. *Bars* with *different letters* indicate significant differences ($P < 0.05$) between two groups

Ziaei-Nejad et al. 2006). In the present study, a better growth performance was obtained from *Bacillus*-fed group. This finding can be attributed to the abundant nutrient content of *Bacillus*, including proteins, minerals and vitamins (Moriarty 1998), which improved the nutritional level of the feed. Additional *Bacillus* in diets can also affect the intestinal microflora of shrimps by increasing the proportion of nonpathogenic bacteria or probiotics, especially *Bacillus. Bacillus* produces a variety of enzymes and other growth-promoting compounds during the fermentation or metabolic process (Rengpipat et al. 1998; Mariel et al. 2004; Wang 2007; Shen et al. 2010), which can offset the insufficient digestive abilities of shrimps caused by high-temperature stress. Consequently, the digestive and absorption abilities of shrimps are improved. Ultimately, the effect of high-water-temperature stress on the physiological metabolism of shrimps can be alleviated, and the growth and survival rates can be improved.

At high temperatures, pathogenic microorganisms in water rapidly reproduce, which may lead to serious bacterial diseases (Atlas et al. 1991; Cheng et al. 2005). In particular, *Vibrio*, a conditioned pathogen, can damage shrimp breeding at high temperatures, often resulting in significant economic losses (Karunasagar et al. 1994; Moriarty 1997; Lavilla-Pitogo et al. 1998). The use of probiotics, with *Bacillus* as the representative, has been considered as an environment-friendly approach to disease control. This method can maintain the flora equilibrium in water or the intestinal microflora balance and inhibit the growth of pathogenic bacteria without the side effects of antibiotics (Verschuere et al. 2000; José et al. 2006). The bacterium can effectively inhibit various Gram-negative and Gram-positive bacteria through nutrition, space competition and production of antimicrobial peptidic substances such as bacitracin, gramicidin S, polymyxin and mixed *Brevibacterium* (Ochoa-Solano and Olmos-Soto 2006). Rengpipat et al. (2000) found that *Bacillus* can be planted in the intestinal tract of *Penaeus monodon* and inhibit the growth of intestinal *Vibrio* through competition, thereby improving the shrimp survival rate. Vaseeharan and Ramasamy (2003) also reported that *B. subtilis* BT23 can effectively control the amount of pathogenic *Vibrio* in the intestinal tract of *P. monodon* and in the breeding water environment. While breeding juvenile *Litopenaeus vannamei* shrimps, José and Tyrone (2007) found that the addition of *B. subtilis* UTM126 can inhibit the growth of *Vibrio* species, such as *Vibrio alginolyticus, Vibrio parahaemolyticus* and *Vibrio harveyi.* Nakayama et al. (2009) found that extracellular substances secreted by *Bacillus* can inhibit *Vibrio* diseases, suggesting that *Bacillus* may affect communication between *Vibrio* cells. The present results also showed that at high-temperature rearing environment, the amount of *Vibrio* in the shrimp intestinal tract and the percentage of *Vibrio* in the heterotrophic bacteria significantly decreased after 15 days of feeding with compound *Bacillus*; this may possibly reduce the infection risk and improve the survival rates of shrimps. In the control group, the amount of *Vibrio* in the shrimp intestinal tract greatly exceeded the threshold of infection, which leads to the low survival rates in shrimp rearing. Of course, the specific mechanism of *Vibrio* inhibition by feeding with *Bacillus* should be researched in depth in the next step.

Under long-term high-temperature conditions, the low immunity of shrimp has been considered as primary cause of slow growth and increased mortality as the physiological and metabolic problems and diseases are frequently observed. In the present study, the dynamic changes in the immunity levels of *M. japonicus* fed with and without *Bacillus* were thoroughly studied. After feeding *Bacillus*, the gene expression level of HcL in hepatopancreas of *M. japonicus* significantly increased ($P < 0.05$). This may indicate that aerobic capacity of shrimps can be enhanced by feeding the animals with *Bacillus*. Under intensive rearing condition, high temperature is a major cause of suffocation for shrimp. By adding additional *Bacillus*, oxygen transmission capacity of shrimp increased, and the risk of insufficient oxygen supply can be decreased. In the present study, aerobic respiration was also enhanced in the *Bacillus*-treated group followed by increasing ROS (Fridovich 1995). Under normal circumstances, the ROS production of organisms is balanced by their antioxidant capability. However, under stressful environments such as high temperatures, this balance will be broken and leads to excessive ROS accumulation. When ROS accumulations pass the safety level, oxidative damage occurs in the organisms (Lopez-Martinez et al. 2008). In this study, the SOD gene expression level in hemocytes of *M. japonicus* significantly increased in the *Bacillus*-fed group ($P < 0.05$). This may indicate that additional *Bacillus* in the diet can protect shrimps from excessive ROS damage under high-temperature stress and improve the antistress ability of shrimp.

In addition to the improved resilience, additional *Bacillus* in the diet can also improve the disease resistance ability of shrimps. Lipopolysaccharides and other substances in the *Bacillus* cell wall can activate the prophenoloxidase (proPO) system and generate phenoloxidase, and other active substances participate in the defensive reaction of the organism through various approaches (Cerenius et al. 2008). This reaction generates opsonin and promotes the phagocytosis, wrapping, tuberculation and solidification of hemocytes while producing bactericidal substances (Söderhäll and Cerenius 1998; Wang and Zhang 2008). In the present study, proPO expression level in the hemocytes of the *Bacillus*-fed group was significantly higher than that in the control group ($P < 0.05$). This finding suggested that feeding shrimp with *Bacillus* in high-temperature environments can enhance the health status and pathogen. Similarly, as an antimicrobial effector molecule (Jollès and Jollès 1984), Lys activity was also increased in the *Bacillus*-fed group in the present study. Further study should be needed to verify the resilience of shrimp against pathogens by feeding shrimp with *Bacillus*.

As an important feed additive, *Bacillus* promoted shrimp immunity and provided more comprehensive nonspecific protection through the competitive inhibition of pathogens. Our present study has successfully demonstrated that additional *Bacillus* in diet can improve growth, survival and immunity of *M. japonicus* reared under high temperature. Although we have provided the details of operating protocols in this article, the actual quantities of *Bacillus* in the diet should be carefully used during the experiment.

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