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Growth Enhancement of Shrimp and Reduction of Shrimp Infection by *Vibrio parahaemolyticus* and White Spot Syndrome Virus with Dietary Administration of *Bacillus* sp. Mk22

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The present study examined the effect of the dietary administration of a halophilic bacterium *Bacillus* sp. Mk22 on the growth improvement of black tiger shrimp, *Penaeus monodon*, and reduced shrimp infection by *Vibrio parahaemolyticus* and white spot syndrome virus (WSSV). The shrimp were fed 45 days using three experimental diets: no addition (control), commercial probiotic, and *Bacillus* sp. Mk22. The shrimp treated with the halophilic bacterium Mk22 showed a significant improvement of growth (7.1 ± 0.21 g), survival (94.3 ± 0.58%), weight gain (178 ± 4.93 g), and reduced feed conversion rate (0.8 ± 0.03 g) compared with the shrimp in the other groups. The shrimp treated with *Bacillus* sp. Mk22 also showed a lower *Vibrio* count (0.02 ± 0.01 × 10^2 CFU/ml) in the shrimp culture water compared with the other groups. The shrimp in the three groups were challenged with either *Vibrio* or WSSV. For the *Vibrio* infection, no mortality was observed from water infection or oral feeding infection in the commercial probiotic group and Mk 22 group. For the WSSV infection, a 68% survival rate from water infection and 20% survival rate from oral feeding infection was observed on day 45 in the Mk22 group, while 100% mortalities were recorded at a much earlier time in both the control and commercial probiotic groups. The antioxidant enzyme activities, indicators of oxidative stress, such as catalase and superoxide dismutase, significantly decreased in both the *Vibrio* and WSSV-infected Mk22 groups compared with the other groups, indicating that *Bacillus* sp. Mk22 was effective in reducing oxidative stress, possibly due to the reduced infection.

Keywords: ***Bacillus* sp. Mk22, *Penaeus monodon*, reduction of infection, *Vibrio parahaemolyticus*, white spot syndrome virus, antioxidant enzymes**

Introduction

The black tiger shrimp *Penaeus monodon* is the most widely cultured shrimp species in Asian countries. However, the shrimp culture industry has recently been badly affected by the outbreak of diseases caused by both pathogenic (virus, bacteria, fungi, and parasites) and non-pathogenic agents (nutritional deficiency, algal toxins, and fluctuating environmental parameters), where the pathogenic forms are responsible for most of the major economic losses. Among the bacterial pathogens, *Vibrio* species cause vibriosis in penaeid shrimp [21], which is mainly caused by *Vibrio parahaemolyticus*, a Gram-negative halophilic, non-spore forming, curved rod-shaped bacterium that naturally lives in estuarine and marine environments worldwide [26]. Meanwhile, the most problematic virus for shrimp cultures around

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the world is the white spot syndrome virus (WSSV), which belongs to the genus *Whispovirus* in the family *Nimaviridae* [16]. WSSV is found in almost all shrimp-producing countries and is lethal to all commercially cultivated Penaeid shrimp species [8, 22].

As chemicals are no longer able to reduce or control the WSSV, alternative methods using probiotic microbes have been attracting attention. Certain halophilic bacteria produce secondary metabolites, which inhibit or kill other harmful microorganisms [1]. Thus, the use of probiotics to enhance the survival, growth, immunity, and disease resistance of farmed aquatic animals is increasing [19].

Infection by *Vibrio* and WSSV causes oxidative stress via the release of reactive oxygen species that are toxic to cells. Shrimp produce a high amount of antioxidant enzymes during a pathogenic infection, which are potential indicators of oxidative stress [17]. Catalase is an important antioxidant enzyme and known to be involved in the crustacean's innate immune reaction [7, 15, 27]. Superoxide dismutase (SOD) is the major O_2 scavenger and its enzymatic action results in H_2O_2 and O_2 formation. Thus, since increased enzyme activity indicates higher stress in the animal, while lower enzyme activity indicates less stress [4], the level of antioxidant enzyme activity could be used as a parameter to measure pathogenic infection.

Accordingly, the present study focused on the effect of *Bacillus* sp. Mk22 (accession number, F794553), a halophilic bacterium previously isolated by the authors [1]. This strain was tested against *Vibrio* and WSSV infection of *P. monodon* under laboratory conditions, as well as on the growth improvement of the shrimp, to explore its possible use as a probiotic.

Materials and Methods

Cultivation of probiotic bacteria and pathogen

Bacillus sp. Mk22 isolated from a saltpan (Tuticorin, India) [1] was used for the *Vibrio* and WSSV infection study. A commercial probiotic (Charoen Pokhpond Aquaculture Pvt. Ltd, India) comprising a *Bacillus* sp. was used for the comparative study. The *Bacillus* cells were cultivated in a trypticase soy broth in a rotary shaking incubator at 37℃ and 200 × *g*. The *V. parahaemolyticus* (laboratory stock culture) was cultured in a nutrient broth supplemented with 3% NaCl at 37℃ and 200 × g. For the *V. parahaemolyticus* specific cell counting medium, a thiosulfate citrate bile salts sucrose agar (TCBS; HiMedia, India) was used.

Shrimp rearing

The *Penaeus monodon* post larvae PL 15 (stage 15) were purchased from the Sona shrimp hatchery, Marakkanam, India and 40 brooders were screened for WSSV infection using the OIE-endorsed nested PCR test [14]. The shrimp were stocked in a 50 L plastic tank containing a 35 L sand-filter and chlorine-treated estuarine water. The postlarvae PL 15 were fed with basic feed at 7.5% body weight per day up to 30 days and then fed at 3.5–5.0% body weight per day. Any unutilized feed was removed 8 hrs after the feeding time.

The growth rate and feed conversion rate (FCR) of the shrimp were calculated using the following equations:

Growth rate (g) = (final body weight – initial body weight) / number of days

FCR (%) = [Feed taken (dry weight in g) / Weight gain (g)] × 100

Shrimp experimental design

The *Penaeus monodon* 4–5 g shrimp (25 shrimp/50 L tank × 3 tanks = 75 shrimp per group) were maintained in separate experimental groups. The control group was fed with basic feed containing no probiotic, the commercial probiotic (CP) group was fed with basic feed containing the commercial probiotic *Bacillus* sp. at a concentration of 1.2×10^8 CFU/ml. The commercial probiotic *Bacillus* sp, was claimed by the manufacturer to have ability to increase the productivity of *Penaeus monodon* and to improve the water quality with decreased concentration of ammonia and nitrite and to control the bacterial infections. The probiotics was also claimed to improve the digestibility of nutrients and to increase the stress tolerance. The Mk22 group was fed with basic feed and *Bacillus* sp. Mk22 at a concentration of 1.2×10^8 CFU/ml. The total numbers of bacteria and *Bacillus* sp. were quantified from the water and shrimp digestive tracts.

Antioxidant enzyme activities

The haemolymph (200 μl) was withdrawn from the haemal sinuses of each shrimp using a 1 ml syringe fit-

ted with a 26-gauge needle, where each syringe was prefilled with 400 µl of an anticoagulant containing 0.94 mM EDTA in an isotonic NaCl solution [6]. The shrimp hepatopancreas tissue (500 mg) was rinsed in ice-cold saline and homogenized with 1 ml 0.15 M Tris-HCl pH 7.4. The haemolymph and hepatopancreas homogenates were then assayed for catalase activity using a colorimetric method [24], and SOD activity using nitroblue tetrazolium [2].

In vivo study

For the *Vibrio* infection, the *V. parahaemolyticus* cells were added to the shrimp culture water at a final concentration of 1.2×10^7 CFU/ml, while the *V. parahaemolyticus* cells mixed with the basic feed at a final concentration of 1.2×10^7 CFU/g were used for oral feeding infection.

For the WSSV infection, a WSSV-infected tissue extract was used. To prepare the tissue extract, 1 g of infected shrimp tissue was homogenized in 9 ml of a TNE buffer (Tris 50 mM, NaCl 100 mM and EDTA 1 mM, pH 7.4) and centrifuged at 4,000 × *g* for 10 min. Thereafter, the supernatant was filtered using a 0.25 µm membrane filter and the resulting clear solution used for the WSSV infection study. A dose of 0.01% WSSV-infected tissue extract was directly added to the shrimp culture water. For oral feeding infection, 500 mg of the WSSV-infected tissue was given to 25 cultured shrimp. Two days before infection, the commercial probiotic and Mk22 groups were both fed with a basic feed pellet mixed with the respective *Bacillus* sp. cells (1.2×10^8 CFU/g). 10% of the water in the shrimp culture tank was exchanged at four-day intervals, and the shrimp behavior was observed every day.

Genomic DNA and PCR amplification

Genomic DNA was isolated from the normal and infected *P. monodon*. The tissue (500 mg) was homogenized using 4.5 ml of a lysis buffer [10 mM Tris (pH 8), 0.1 M EDTA, 20 µg/ml pancreatic RNAase, 0.5% SDS], treated with proteinase K (0.2 mg/ml) and sarkosyl (1%) at 45℃ for 2–4 h, followed by phenol-chloroform extraction and dialysis against a TE buffer [20]. Two pairs of oligonucleotide primers of 22 bp and 21 bp named as LoF1, LoR1 and LoF2, LoR2 (PROLIGO Primers & Probes, Australia) specific for WSSV DNA sequences [3] were used. The PCR reaction kit (Boehringer Mannheim, Germany) and reaction conditions were initial denaturation at 94℃ for 7 min, followed by 35 cycles of 94℃ for 30 s, 52℃ for 30 s, 68℃ for 1 min, and the final extension was 72℃ for 10 min. The 1.2% agarose gels were visualized using UV transillumination (Gel doc 2000, Japan).

Statistical analysis

All the statistical data with means were compared using Duncan's multiple range tests (SPSS 16 software, IBM, USA). A significance level of $p \leq 0.05$ was used for all tests. The data are reported as the means ± standard deviation. The different letters on the same patterned/colored bars of the charts indicate a statistical difference among the experiments ($p < 0.05$).

Results

Growth and FCR

The growth rate (7.1 ± 0.21 g) and total weight (178 ± 4.93 g) were the highest in the Mk22 group (on day 45). The CP group showed a lower growth rate (5.8 ± 0.20 g) and total weight (147.7 ± 5.69 g), and the control group showed the lowest growth rate (3.6 ± 0.20 g) and total weight (90.7 ± 5.51 g). A high FCR of 1.32 ± 0.09% was recorded in the control group, a lower rate of 1.00 ± 0.09% in the CP group, and the lowest of 0.80 ± 0.03% in the Mk22 group. Therefore, *Bacillus* sp. Mk22 showed the best results for the growth rate and FCR.

Bacterial count

The highest total *Vibrio* count (TVC) $4.97 \pm 0.053 \times 10^2$ CFU/ml was in the control group, with a low count of $0.2 \pm 0.01 \times 10^2$ CFU/ml in the CP group, and the lowest of $0.02 \pm 0.01 \times 10^2$ CFU/ml in the Mk22 group observed

Table 1. Total bacterial and *Bacillus* counts in water and digestive tracts of different groups.

Group	Water		Digestive track	
	Total count ($\times 10^5$ CFU/ml)	*Bacillus* count ($\times 10^4$ CFU/ml)	Total count ($\times 10^5$ CFU/g)	*Bacillus* count ($\times 10^4$ CFU/g)
Control	25.3 ± 1.52[a]	0.0 ±-	35.0 ± 1.16	0.0 ± -
CP	22.3 ± 0.73	10.4 ± 0.65	9.5 ± 0.76	4.6 ± 0.56
Mk22	21.7 ± 0.58	16.1 ± 1.01	4.2 ± 0.42	7.4 ± 0.65

[a]Mean ± Standard deviation.

on day 45 of culturing (data not shown). *Bacillus* sp. Mk22 showed the highest number in both the culture water and the digestive tract of the shrimp (Table 1) ($p \leq 0.05$), and seemed to be well colonized in the digestive tract compared to the other groups.

Mortality of *P. monodon* infected with either *V. parahaemolyticus* or WSSV

The mortality of the shrimp challenged with *V. parahaemolyticus* was examined. For the water infection of the control group, 2 out of 25 shrimp had died by day 15, while the maximum mortality of 12 was reached on day 25. For the oral feeding infection of the control group, 1 shrimp had died by day 9, while the maximum mortality of 8 was reached on day 20. Therefore, the water infection produced a higher mortality. For both the CP and Mk22 groups, no mortality was recorded from either the water or the oral feeding infection (data not shown). Therefore, the *Bacillus* sp. used for the CP and Mk22 groups were significantly effective in reducing the shrimp mortality by *V. parahaemolyticus*.

For the WSSV water infection, 100% mortality was reached on day 15 in both the control and CP groups (Fig. 1A). In the Mk22 group (water infection), 68% of the shrimp were still alive on day 45 (Fig. 1A). For the oral feeding infection, 100% mortality was reached on day 8 and 10 in the control and CP group, respectively (Fig. 1B). In the Mk22 group (oral feeding infection), 20% of the shrimp were still alive on day 45 (Fig. 1B). The surviving animals were tested using a 2-step PCR and the results revealed they were WSSV positive. Therefore, the above results indicate that the dietary administration of *Bacillus* sp. Mk22 was strongly effective in reducing the shrimp mortality by WSSV, espe-

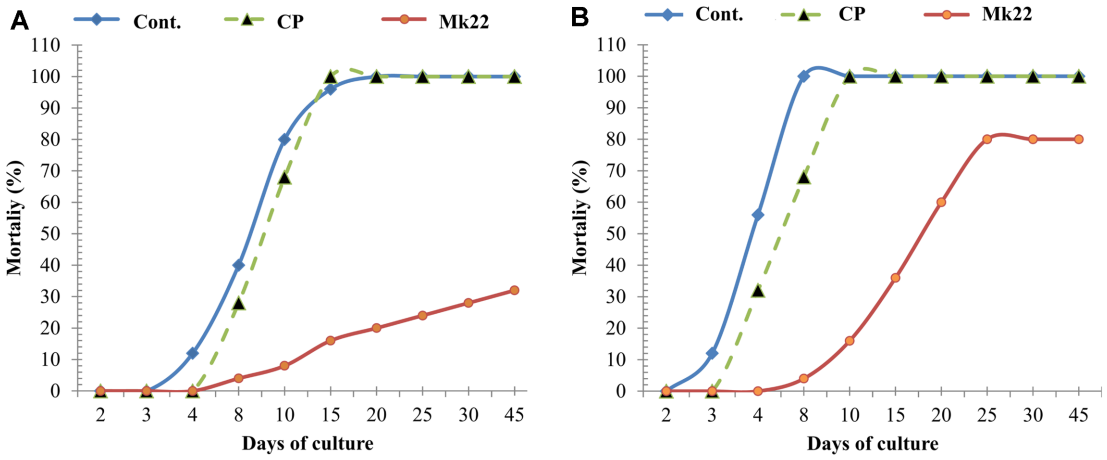

Fig. 1. Mortality of WSSV-infected *P. monodon* in water (A) and oral (B) infection.

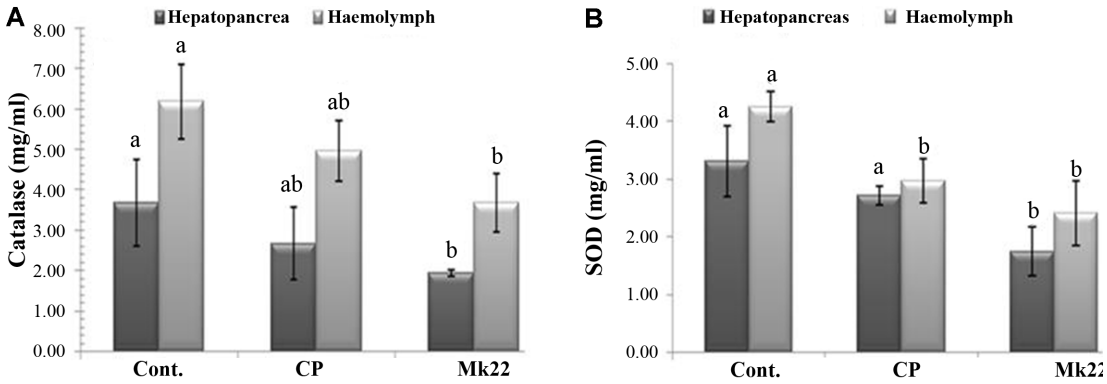

Fig. 2. Catalase (A) and SOD (B) activity of *Vibrio*-infected hepatopancreas and haemolymph in *P. monodon*.

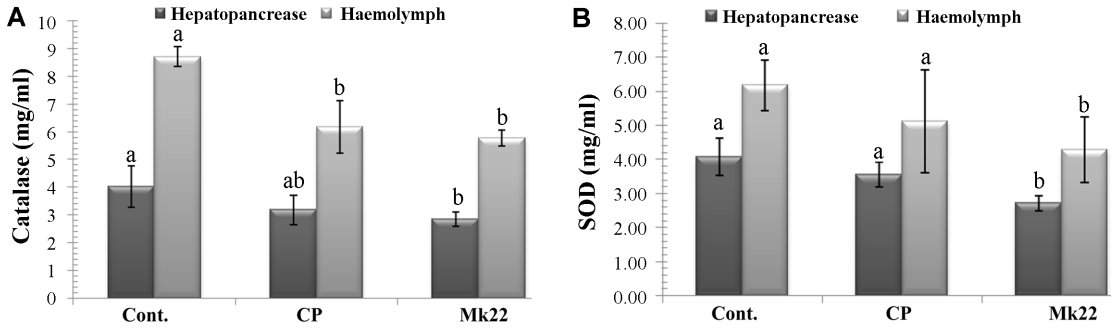

Fig. 3. Catalase (A) and SOD (B) activity of WSSV-infected hepatopancreas and haemolymph in *P. monodon*.

cially by water infection.

Catalase and SOD activity in either *Vibrio* or WSSV-infected hepatopancreas and haemolymph

In the *V. parahaemolyticus* infection experiments, the highest catalase and SOD activities were observed in the control group, median activities in the CP group, and the lowest activities in the Mk 22 group (Fig. 2A, B). The catalase and SOD activities were higher in the haemolymph than in the hepatopancreas for all three groups.

The WSSV infection experiments also showed similar results to the experiments with *V. parahaemolyticus*; for both the hepatopancreas and the haemolymph, the highest catalase and SOD activities were observed in the control group, median activities in the CP group, and the lowest activities in the Mk 22 group (Fig. 3A, B). Therefore, the Mk22 group exhibited the least catalase and SOD activities in both the *V. parahaemolyticus*-infected and WSSV-infected shrimp when compared to the other experimental groups, indicating that *Bacillus* sp. Mk22 contributed to reducing the catalase and SOD activities in both the hepatopancreas and the haemolymph of the infected shrimp.

Discussion

In shrimp cultures, vibriosis causes more than 70% mortality within one day after infection [12]. Plus, WSSV is one of the most serious viral pathogens for shrimp; 100% mortality can be reached within a short span of time (7 to 10 days) [13]. A growing number of studies have demonstrated the use of probiotics in aquaculture and their ability to control potential pathogens, while also increasing the growth rates and welfare of the farmed aquatic animals [25]. Thus, the present study attempted to enhance the growth rate of *P. monodon* supplemented with the bacterial isolate *Bacillus* sp. Mk22, while also reducing the infection in the *P. monodon* culture. The results showed a significantly increased final weight compared with the cultures supplemented with a commercial probiotic or without probiotic supplementation. Plus, *Bacillus* sp. Mk22 facilitated a high survival rate of 100% during the *Vibrio* challenge experiment, while *Pediococcus acidilactici* in another report only exhibited a 67% survival [4].

Some of the reasons for the enhanced shrimp growth could be higher activities of digestive enzymes, like amylase, protease, and lipase, induced by the probiotics [11, 28] and high probiotic bacteria in the digestive tract [28], along with the infection-reducing effect of the probiotics [4]. Enhanced resistance to pathogens occurs by activating both cellular and humoral immune responses in shrimp [19], and *Bacillus* surface antigens or their metabolites act as immunogens for shrimp by stimulating the phagocytic activity of granulocytes [9]. Halophilic bacteria also produce several secondary metabolites, such as bacteriocins, bacteriocin-like substances, and antibacterial lipopeptides against pathogens [11].

When *P. monodon* was challenged with WSSV, the results revealed that 68 and 20% of the shrimp survived in the Mk 22 group following water infection and oral feeding infection, respectively, whereas 100% mortality was recorded at a much earlier time in both the control and CP groups. Therefore, *Bacillus* sp. Mk22 significantly reduced the mortality compared with the other groups.

When shrimp are infected, oxidative stress that produces a high amount of antioxidant enzymes, like cata-

lase and SOD, is also increased [15]. Catalase and SOD are important antioxidant enzymes, present in almost all oxygen-respiring animals. Thus, in this study, the hepatopancreas and haemolymph were used to measure the change in catalase and SOD activities. The hepatopancreas is the main organ for the reserve and detoxification of xenobiotics in crustaceans, and is highly sensitive to physiological and environmental changes [10]. Plus, the catalase and SOD activities were also measured in the haemolymph, since the blood cells of invertebrates are the primary effectors in the host defense and involved in various immune processes, such as phagocytosis [23].

The level of these enzymes in the Mk22 group was significantly decreased in both the *V. parahaemolyticus*- and WSSV-infected *P. monodon* hemolymph and hepatopancreas when compared with the other groups. In the Mk22 group, the lowered catalase and SOD activities seemed to be due to less stress caused by reduced infection as a result of the administration of *Bacillus* sp. Mk22. These results concur with the finding of Chang *et al.* [5], where SOD activity decreased in WSSV-infected *P. monodon* with probiotic administration. Castex *et al*. [4] also reported that in shrimp exposed to *Vibrio,* the antioxidant response was characterized by higher antioxidant enzyme activities (catalase and SOD) and a higher oxidative stress level compared to the levels found in the control without *Vibrio*. We also found that the shrimp fed with the probiotic diet exhibited a lower prevalence of *Vibrio* throughout the test in the digestive tract, plus the antioxidant response and oxidative stress level recorded in the digestive gland of the shrimp fed with the probiotic diet were lower.

Therefore, based on the above observations, it was concluded that the strain of halophilic *Bacillus* sp. Mk 22 was effective in inhibiting infection by shrimp pathogens, such as *V. paraheamolyticus* and WSSV. The bacterium significantly reduced the mortality and did not have any pathogenic effect on the shrimp. Indeed, the *P. monodon* exhibited a significantly higher weight gain and survival ratio than the control group. Therefore, *Bacillus* sp. Mk 22 could be used effectively to control such shrimp pathogens and enhance shrimp production, thereby substituting for the use of antibiotics in aquaculture.

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국문초록

Bacillus sp. Mk22의 섭취가 _Penaeus monodon_ 새우의 성장증진과 _Vibrio parahaemolyticus_와 흰반점바이러스(WSSV)의 감염 감소에 미치는 영향

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본 연구에서는 호염성 세균 *Bacillus* sp. Mk22를 사료에 섞어 투여함으로써 *Penaeus monodon* 새우의 성장증진과 *Vibrio parahaemolyticus*와 흰반점바이러스(WSSV)의 감염 감소에 대한 영향을 조사하였다. 새우는 45일 동안 3가지 먹이를 공급하였는데, 무첨가(대조구), 시판 프로바이오틱, 그리고 *Bacillus* sp. Mk22이었다. 호염성세균 Mk22를 투여한 새우들은 다른 그룹들의 새우들보다 성장(7.1 ± 0.21 g), 생존율(94.3 ± 0.58%), 체중증가(178 ± 4.93 g), 그리고 감소된 사료전환효율(0.8 ± 0.03 g)을 보였다. *Bacillus* sp. Mk22를 투여한 새우들은 다른 그룹의 새우들보다 새우배양 수조안에서 낮은 비브리오 수자($0.02 \pm 0.01 \times 10^2$ CFU/ml)를 보였다. 이 세 그룹의 새우들을 비브리오나 WSSV로 공격감염을 하였다. 비브리오 감영에서는, 시판 프로바이오틱 그룹과 Mk22 그룹에서 배양수조의 물 감염과 경구투여감염에서 치사 새우가 없었다. WSSV 감염에 대해서는, Mk22 그룹의 경우 새우배양 수조 감염에서는 68%의 생존율과 경구투여 감염에서는 20% 생존율이 45일째에 관찰되었고, 대조구와 시판 프로바이오틱 그룹에서는 모두 훨씬 빠른 시간 내에 100% 사망률을 보였다. 산화적 스트레스의 표지인, catalase와 superoxide dismutase와 같은 항산화 효소 활성은 비브리오와 WSSV 감염된 Mk22 그룹 모두에서 다른 그룹들에 비해 유의적으로 감소하였다. 이 사실은 *Bacillus* sp. Mk22는 산화적 스트레스 감소하는데 효과적이었고, 이는 감소된 감염효과로 인한 것으로 추정된다.