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Efficacy of a probiotic *Bacillus subtilis* strain in fish culture water for ammonia removal and enhancing survival of juvenile common carps (*Cyprinus carpio*)

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Abstract Total ammonia nitrogen (TAN) was significantly reduced by treatments of 10.0 and 5.0 mg/L probiotic *Bacillus subtilis* within 48 hours ($p<0.05$). Fish, common carp (*Cyprinus carpio*), survival in all treatment groups was higher than the control group significantly ($p<0.05$). *B. subtilis* concentration of 5.0 and 10.0 mg/L can reduce ammonia in fish culture water and promote fish survival. This *B. subtilis* strain should be an optional probiotic in fish culture.

Keywords: Total ammonia nitrogen (TAN), Probiotic, *Bacillus subtilis*, Common carp (*Cyprinus carpio*)

Introduction

At present, most aquaculturists try to produce more fish from their farms; however, if there is no good management practice in the aquaculture farm, water pollution occurs. One example is overfeeding of aquatic animals that can lead to uneaten food and overexcretion which gradually generates wastes such as ammonia and others (Tomasso, 1994). Unionized ammonia (NH_3), an important nitrogenous waste resulting from a metabolism of protein, can be harmful to aquatic animals. A high NH_3 concentration can cause many problems to aquaculture such as low quality of water and weaken aquatic animals' health, which these might cause microbe infections leading to the death of the said animals (Assefa and Abunna, 2018). NH_3 is released from animals' gills which are 2.5-3% of the total daily diet (Lawson, 1995). Even though the toxicity of ammonia can be got rid of by replacing water; however, this way is not guaranteed to remove all ammonia (Moeckel *et al*., 2012). In high density

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aquaculture, if water is not managed well, ammonia may rise in a high level and cause danger to the aquatic animals. Ammonia causes chronic toxicity to the animals' health rather than causing immediate mortality (Hargreaves, 1998). When water contains high concentration of ammonia, the aquatic animals will excrete less ammonia, therefore increasing the ammonia levels in their blood and tissues which would then lead to high pH of blood that affects enzymes in their body. A long term of ammonia exposure toxicity has an impact on reducing growth rate and increasing feed conversion ratio. High levels of ammonia also damage animals' gills by causing gingivitis resulting in low oxygen exchange. Moreover, ammonia might block the ability of red blood cells to transport oxygen to the body tissues which could cause lack of oxygen on the aquatic animals and therefore diminishes their resistance to diseases (Wedemeyer *et al*., 1976).

Fish can tolerate a certain amount of ammonia as reported by Abbas (2006) which showed the 96-hour exposure LC_{50} toxicity of ammonia on common carp fingerlings, *Cyprinus carpio,* obtained an amount of 0.93 mg/L at pH 7.5 (Abbas, 2006). High level of ammonia in water makes the fish excrete less ammonia which then leads to high pH in the fish's blood. Its high level has also an effect on water exchange, so the concentration of minerals in fish's bodies is decreased and also the ability of blood cells to transport oxygen are lower. These weaken their resistance to diseases and reduce growth rate (Colt and Armstrong, 1979). However, fishes are vertebrates; they have a harmless process to eliminate ammonia since the latter performs a chemical reaction with glutamic acid in their blood and turns to glutamine. When blood flows to kidneys, it will be filtered and ammonia is separated as a form of waste. Therefore, fishes have a higher ability to resist ammonia than other invertebrates such as shrimps or crabs (Boyd, 2013).

Ammonia can be treated by the nitrification process which is a part of the natural nitrogen cycle. This cycle uses nitrifying bacteria that get their energy from the oxidation of inorganic *Nitrosomonas* spp. and change ammonia to nitrite (NO_2^-) according to the following equation (Hagopian and Riley, 1998).

$$NH_4^+ + O_2 \rightarrow NO_2^- + 2H^+ + H_2O$$

Apart from that, nitrifying bacteria can oxidize ammonia which is very dangerous to aquatic animals to nitrite, but nitrate is low toxicity. This process could occur in the best condition when pH is at 7-8 and temperature is at 25-35°C. If pH is higher, ammonia will be in NH_3 form that generates toxicity to aquatic life. Although ammonium (NH_4^+) is also poisonous, the level of toxicity is 50% lower than ammonia (Meade, 1985). Moreover, denitrifying bacteria that include species of genera such as *Pseudomonas stutzeri, Thioalkalivibrio* *denitrificans, Paracoccus denitrificans, Bacillus* sp. and *Alcaligenes* sp. can oxidize nitrite and nitrate by the denitrification process. After an oxidation, nitrogen gas (N_2) is released and returns to the atmosphere according to the following equations (Lee *et al.,* 2000):

$$2NO_2^- + 6H^+ + 6e^- \rightarrow N_2 + 2HO + 2H_2O$$

$$2NO_3^- + 10H^+ \rightarrow N_2 + 2HO + 4H_2O$$

At present, probiotic microorganisms are widely used to control ammonia in aquaculture (Hong *et al.,* 2005). There are bacterial groups such as nitrifying bacteria which can oxidize ammonia to nitrite or nitrate that has lower toxicity, and denitrifying bacteria which can oxidize nitrite or nitrate and reverse it to be nitrogen (Hong *et al.*, 2005). Moreover, the probiotics used to control ammonia should grow fast, generate spores in order to be resistant to environments, be harmless to aquatic animals, and can produce bioactive compounds to improve the quality of water and boost the immune system of aquatic animals (Hong *et al.,* 2005). *Bacillus* spp. is appropriate to be probiotics in aquaculture because they can generate spores (Hong *et al.,* 2005). Some species such as *B. flexus, B. subtilis, B. cereus,* and *B. licheniformis* are able to reduce ammonia and nitrite by employing nitrification and denitrification processes (Kim *et al.*, 2005). There are many reports showed using *Bacillus* spp. to reduce ammonia resulting in improving water quality in aquaculture (Xie *et al.*, 2013; Zokaeifar *et al.*, 2014; Elsabagh *et al.,* 2018; Hlordzi *et al.*, 2020) and promoting survival of aquatic animals (Xie *et al.,* 2013; Songsuk *et al.,* 2018; Tarnecki *et al.*, 2019). In Thailand, Songsuk *et al.* (2018) reported *B. subtilis* can increase survival rate of Pacific white shrimp (*Litopenaeus vannamei*). However, there is no previous report yet about application of *Bacillus* species to control ammonia in fish culture and enhance fish survival in Thailand. Consequently, this study aimed to investigate the efficacy of a probiotic *B. subtilis* on reducing ammonia in fish culture water, and promoting the survival of juvenile common carps (*Cyprinus carpio*).

Materials and methods

Experiment plan

The completely randomized design (CRD) was employed with 3 replicates and 4 treatments as the following: control group (no probiotic), treatment 1: probiotic concentration of 2.5 mg/L in water, treatment 2: Probiotic concentration of 5.0 mg/L in water, and treatment 3: Probiotic concentration of 10.0 mg/L in water.

Probiotic preparation

The lyophilized powder 'Biopondplus®' of a probiotic *B. subtilis* strain (White Crane V.88 Aqua-Tech Company Limited) was mixed in fish culture water according to the treatments. To examine the amount of probiotic, colony count (CFU/mL) was performed after applying the probiotic for 24 hours. *Bacillus* species was confirmed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS, Bruker Daltonics).

Water quality control

Water quality in the fish culture was controlled to 7.0-8.5 pH, a temperature of 25-30°C, sterilized with 20 ppm chlorination, and eliminated chlorine using an aerator in the 500 L tanks. Water was supplied into 12 tanks by 50 L in each. During the experiment, water quality was measured daily in the morning using a water test kit for pH (7.0-8.5), dissolved oxygen (>5ppm), and dissolved nitrite (<0.75 ppm). There was no water change during the experiment period.

Fish preparation

A total of 260 common carp juveniles (2 inches in length) were obtained from a commercial farm. There were 2 tanks used, fish were acclimatized in the 500 L tanks containing 130 common carp juveniles in each. During 3 days of acclimation, fish were fed twice a day, in the morning and evening (8-10% of the total fish weight in each meal) by instant food containing approximately 35% of crude protein. The prepared fish were then randomly distributed into 12 separate tanks (24” x 10” x 12”) with 20 fish in each.

Data collections

Water in each tank was collected for 30 mL daily at 9 a.m. and measured the total ammonia nitrogen (TAN) concentration using Multimode Microplate Reader (EnSight™). The experiment period lasted for 8 days, so water was collected at 0h, 24, 48, 72, 96, 120, 144, 168, and 192 hours, respectively. The survival rate of fish was examined by counting dead fish in each tank daily in the morning.

Statistical analysis

TAN concentration and survival rate of fish were analyzed by One-way ANOVA test and Pearson's chi-squared test, respectively, with a confidential level of 95% using IBM SPSS version 22.0.

Results

Colony count

B. subtilis concentration after adding the probiotic at 24 hours is shown on Table 1. The adding of probiotic concentration of 0.00, 2.5, 5.0, and 10.0 mg/L yielded *B. subtilis* concentration of 0.00, 4.94 x 10^{5}, 9.31 x 10^{5}, and 1.82 x 10^{6} CFU/mL, respectively.

Table 1. *B. subtilis* colony count yielded by different probiotic concentration within 24 hours

	Probiotic concentration (mg/L)	***B. subtilis* colony count (CFU/mL)**
Control group	0.0	0.00
Treatment 1	2.5	4.94 x 10^{5}
Treatment 2	5.0	9.31 x 10^{5}
Treatment 3	10.0	1.82 x 10^{6}

Table 2. TAN concentration in water at different times

Time (hours)	**TAN concentration (mg/L; mean ± S.D.)**				***p*-value**
	Control group	**Treatment groups (probiotic concentration)**			
		2.5 mg/L	**5.0 mg/L**	**10.0 mg/L**	
0	0.0125 ± 0.0001	0.0124 ± 0.0001	0.0126 ± 0.0001	0.0125 ± 0.0002	0.452
24	0.0125 ± 0.0001	0.0125 ± 0.0001	0.0126 ± 0.0001	0.0125 ± 0.0002	0.723
48	0.0123 ± 0.0001^{a}	0.0120 ± 0.0000^{b}	0.0112 ± 0.0000^{c}	0.0114 ± 0.0004^{c}	0.000^{*}
72	0.0124 ± 0.0001^{a}	0.0118 ± 0.0000^{b}	0.0087 ± 0.0001^{c}	0.0112 ± 0.0001^{c}	0.000^{*}
96	0.0124 ± 0.0000^{a}	0.0115 ± 0.0001^{b}	0.0070 ± 0.0001^{c}	0.0112 ± 0.0000^{c}	0.000^{*}
120	0.0131 ± 0.0001^{a}	0.0112 ± 0.0002^{b}	0.0057 ± 0.0000^{c}	0.0077 ± 0.0001^{c}	0.000^{*}
144	0.0132 ± 0.0001^{a}	0.0112 ± 0.0003^{b}	0.0058 ± 0.0001^{c}	0.0064 ± 0.0001^{c}	0.000^{*}
168	0.0150 ± 0.0010^{a}	0.0113 ± 0.0001^{b}	0.0058 ± 0.0000^{c}	0.0065 ± 0.0000^{c}	0.000^{*}
192	0.0155 ± 0.0009^{a}	0.0109 ± 0.0002^{b}	0.0059 ± 0.0000^{c}	0.0066 ± 0.0001^{c}	0.000^{*}

Note: Values with different superscripts in the same column indicate a significant difference ($p<0.05$).

TAN concentration

Treatment groups of 2.5, 5.0, and 10.0 mg/L showed TAN concentration was significantly lower than the control group during 48-192 hours ($p<0.05$) as shown on Table 2. Moreover, the treatments of 5.0 and 10.0 mg/L showed TAN concentration was lower than the treatment of 2.5 mL during 48-192 hours ($p<0.05$).

Fish survival rate

No mortality was observed in the experiment until 24 hours; all groups showed the fish survival rate of 95% in each (Table 3). At 192 hours, the fish survival rate in the treatments of probiotic 2.5, 5.0, 10.0 mg/L, and the control group was 88.33, 91.67, 90.00, and 85.00%, respectively. All treatment groups showed the fish survival rate was significantly higher than the control group ($p<0.05$).

Table 3. Fish survival rate at different times

Time (hours)	Fish survival rate (%)			
	Control group	Treatment groups (probiotic concentrations)		
		2.5 mg/L	5.0 mg/L	10.0 mg/L
0	100.00	100.00	100.00	100.00
24	95.00	95.00	95.00	95.00
48	93.33	90.00	91.67	90.00
72	93.33	90.00	91.67	90.00
96	88.33	90.00	91.67	90.00
120	85.00	88.33	91.67	90.00
144	85.00	88.33	91.67	90.00
168	85.00	88.33	91.67	90.00
192	85.00	88.33	91.67	90.00
p-value		0.008*	0.020*	0.021*

Note: Values with * indicate a significant difference ($p<0.05$).

Discussion

Using probiotic concentrations of 2.5, 5.0, and 10.0 mg/L could reduce ammonia in fish culture water of common carp, *Cyprinus carpio*, during 48-192 hours. These treatment groups yielded *B. subtilis* concentration of 10^5-10^6 CFU/mL. The results were similar to the report of Zokaeifar *et al*. (2014) showing 10^5 CFU/mL of *B. subtilis* can reduce ammonia significantly in the rearing water of shrimp, *Litopenaeus vannamei*. Whereas, previous reports showed 10^8-10^9 CFU/mL of *B. subtilis* can reduce ammonia in fish culture, such as a study of Zhang *et al.* (2013) used 10^9 CFU/mL *B. subtilis* to reduce ammonia in grass carp (*Ctenopharyngodon idellus*) culture water, while Elsabagh *et al.* (2018) and Mohammadi *et al.* (2020) used 10^8-10^{10} CFU/g *Bacillus* spp. mixed in a fish diet could reduce ammonia in fish culture water significantly. However, this study showed using higher probiotic concentrations (5.0 and 10.0 mg/L) can significantly reduce ammonia better than the reported lower concentration (2.5 mg/L).

B. amyloliquefaciens can reduce 20 mg/L of ammonia in water effectively when using 10^8 CFU/mL of *B. amyloliquefaciens* at 30°C and pH 8 (Xie *et al*., 2013). However, this study showed 10^5-10^6 CFU/mL of *B. subtilis* can approximately reduce 0.01 mg/L ammonia. While in this study, ammonia level was lower considering the measurement of ammonia in fish culture water; whereas Xie *et al.*'s reports was performed in simulating water without aquatic fauna. The ammonia level of 0.1-10 mg/L in water can cause 50% mortality (LC_{50}) of a number of fish and shrimp (Philips *et al*., 2007). In addition, ammonia can be toxic to commercially cultured fish at concentrations above 1.5 mg/L (Crab *et al*., 2007). There has not been any studied on the potential application of *B. subtilis* in removing ammonia in fish culture water; thus, this study is the first and consequently suggests that *B. subtilis* might be interested in alternative probiotic for ammonia removal.

Bacillus species are interesting microorganisms for developing commercial probiotics for ammonia removal and water quality enhancement (Hong *et al.,* 2005). Previous reports showed *B. cereus* (Lalloo *et al*., 2007), *B. licheniformis* (Meng *et al*., 2009), and *B. subtilis* (Meng *et al.,* 2009; Chen and Hu, 2011) can reduce ammonia in aquaculture. *Bacillus* spp. could utilize nitrate and nitrite as alternative electron acceptors and nitrogen sources (Nakano *et al*., 1998; Hoffmann *et al*., 1998). This study showed a *B. subtilis* strain, isolated from farming shrimp, can also reduce ammonia in fish culture water within 48 hours. Previously, *Bacillus* spp. were applied in enhancing water quality (Lalloo *et al.,* 2007; Meng *et al*., 2009; Chen and Hu, 2011; Xie *et al*., 2013; Zokaeifar *et al*., 2014; Elsabagh *et al.,* 2018; Hlordzi *et al*., 2020), and promoting growth performance (Zokaeifar *et al.,* 2014; Elsabagh *et al.,* 2018; Mohammadi *et al.*, 2020; Rahman *et al*., 2021; Van Doan *et al*., 2021), immune response (Zokaeifar *et al*., 2014; Mohammadi *et al.*, 2020; Rahman *et al.,* 2021; Van Doan *et al*., 2021), blood profile, intestinal morphology (Elsabagh *et al.,* 2018), survival rate (Songsuk *et al.,* 2018; Tarnecki *et al.,* 2019), and microbial resistance against pathogenic bacteria such as *Vibrio* spp. (Xie *et al*., 2013; Songsuk *et al*., 2018; Mohammadi *et al.,* 2020; Rahman *et al*., 2021; Van Doan *et al*., 2021) of fish and shrimps.

In this study, there was a satisfying score of fish survival—high rate of survival—as all treatment groups showed significantly higher rate than the control group. Previous studies confirmed that *Bacillus* species have the potential to improve fish survival (Songsuk *et al*., 2018; Tarnecki *et al*., 2019). In the present study, the use of 10^5-10^6 CFU/mL of *B. subtilis,* which was lower than that of Won *et al.* (2020) used 10^8 CFU/mL of *B. subtilis,* mixed in fish diet could significantly enhance survival of the Nile tilapia (*Oreochromis niloticus*). Moreover, Tarnecki *et al.* showed *B. licheniformis* and *B. amyloliquefaciens* can enhance 20% higher survival rate of common snook (*Centropomus undemicalis*) at 7 days following its transportation (Tarnecki *et al.,* 2019). Songsuk *et al.* (2018) reported similarl that *B. subtilis* can promote survival of Pacific white shrimp (*Litopenaeus vannamei*) larvae from the pathogenic *Vibrio* species. The abovementioned reports exhibited *Bacillus* species can promote fish survival regardless of stressful and pathogenic conditions, respectively. In conclusion, the study suggested that *B. subtilis* commercial strain could be applied as an alternative probiotic for ammonia removal in fish culture water as well as enhancing the fish survival. The probiotic 5.0-10.0 mg/L yielding 10^5-10^6 CFU/mL could reduce ammonia in fish culture water within 48 hours of application. However, the appropriate probiotic concentration for enhancing fish survival should be performed for further studies.

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