

Effect of Dietary Organic Acids and Astaxanthin on Growth, Survival and Tolerance to *Vibrio* Infection in Pacific White Shrimp (*Litopenaeus vannamei*)

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ABSTRACT

A study on the effect of organic acid (AMASIL NA, Formic acid 94%) and astaxanthin (Lucantin Pink CWD, Astaxanthin 10%) on growth and survival of Pacific white shrimp (*Litopenaeus vannamei*) was carried out under laboratory conditions. Tests with six treatments (four replicates/treatment) were conducted. Postlarvae (PL12) were stocked in 500-L fiberglass tanks (salinity 25 ppt, temperature $29 \pm 1^{\circ}\text{C}$) at a density of 75 PL/tank. Shrimp were fed four times daily with pelleted feed containing AMASIL NA at 0.3 %, AMASIL NA at 0.6 %, Lucantin Pink CWD 50 ppm, AMASIL NA at 0.3 % + Lucantin Pink CWD 50 ppm, AMASIL NA at 0.6 % + Lucantin Pink CWD 50 ppm and the control group (without AMASIL NA and Lucantin Pink CWD). After 60 days of dietary administration, shrimp fed with Lucantin Pink CWD 50 ppm had an average body weight of 4.45 ± 0.45 g, which was higher than those of shrimp fed with AMASIL NA at 0.3 % + Lucantin Pink CWD 50 ppm (4.38 ± 0.37 g), AMASIL NA at 0.6 % + Lucantin Pink CWD 50 ppm (4.05 ± 0.21 g) and the control group (4.18 ± 0.05 g). There was a statistical difference between these groups and those fed with AMASIL NA at 0.3 % (3.68 ± 0.49 g) and AMASIL NA at 0.6 % (3.88 ± 0.22 g). Shrimp fed with AMASIL NA at 0.6 % + Lucantin Pink CWD 50 ppm had the highest survival rate of 82.33 ± 8.32 %, followed by those fed with AMASIL NA at 0.3 % + Lucantin Pink CWD 50 ppm (78.67 ± 7.77 %), Lucantin Pink CWD 50 ppm (78.33 ± 9.26 %), AMASIL NA at 0.3 % (72.33 ± 9.94 %) and AMASIL NA at 0.6 % (67.67 ± 14.29 %). Survival rates of these groups were significantly different from the control group (64.33 ± 10.12 %). A study of the effects of AMASIL NA and Lucantin Pink CWD on the prevention of *Vibrio parahaemolyticus* outbreak in rearing Pacific white shrimp was conducted under laboratory conditions. Thirty shrimp (8-9 g) from each tank in the previous experiment were randomly sampled and stocked in 12 x 500-L fiberglass tanks with four replicate tanks per treatment. *V. parahaemolyticus* were added into each tank to obtain final concentration of 10^4 CFU/ml. Each treatment group received aforementioned diets four times daily for 30 days. At the end of the trial, there

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were no significant differences among the average body weights of shrimp from the six experimental groups. However, shrimp fed with AMASIL NA at 0.6 % + Lucantin Pink CWD 50 ppm had the highest survival rate ($67.50 \pm 3.33\%$), followed by those fed with AMASIL NA at 0.6 % ($65.00 \pm 5.77\%$), Lucantin Pink CWD 50 ppm ($56.67 \pm 17.11\%$) and AMASIL NA at 0.3 % + Lucantin Pink CWD 50 ppm ($54.17 \pm 5.09\%$). There were statistical differences between these groups and those fed with AMASIL NA at 0.3 % ($45.83 \pm 16.78\%$) and the control group ($20.00 \pm 17.32\%$). Shrimp fed with AMASIL NA showed significantly lower number of *Vibrio* spp. in the intestine compared to the group fed with Lucantin Pink CWD 50 ppm and the control group.

Keywords: Pacific white shrimp, Organic acid, Astaxanthin

INTRODUCTION

Currently, Pacific white shrimp, *Litopenaeus vannamei*, native to the Pacific coasts of Central and South America, is the major shrimp species cultured in China, Taiwan and Thailand (Limsuwan and Chanratchakool, 2004). Since 2012, shrimp farmers in Thailand have experienced Early Mortality Syndrome (EMS) which caused major economic losses in many shrimp culture areas throughout the country. Affected shrimp show signs of pale coloration due to pigment loss as well as atrophy of the hepatopancreas. These signs may become apparent as early as 4 days post stocking (Munkongwongsiri *et al.*, 2013). *Vibrio parahaemolyticus* was reported as the suspected agent causing the mass mortality (Tran *et al.*, 2013). Acidification of animal and aquaculture feeds by adding organic acids and organic salts can inhibit the growth of bacteria and fungi in the intestinal tract (Iba and Berchieri 1995; Franco *et al.* 2005). The improvement in the balance of gut microfloral communities can enhance the digestibility of some proteins and lipids

leading to improvements in weight gain and survival (Eidelsburger *et al.*, 1992; Roth *et al.*, 1992; Hansen *et al.*, 2007; Castro, 2005). Although organic acids and organic acid salts have been wildly used in terrestrial livestock feeds, studies in marine shrimp nutrition are limited (Walla *et al.*, 2012; Salem and Amin, 2012). Astaxanthin is the main carotenoid found in the integument of shrimp. It improves health and immunology through the removal of oxygen free radicals produced from normal cellular activity and various stressors (Chew, 1995; Supamattaya *et al.*, 2005). Survival improvement in marine shrimp by dietary astaxanthin supplementation has been reported by Chien and Jeng (1992) and Thongrod *et al.*, (1995). Thus the objective of this study was to evaluate the use of dietary supplementation of organic acid, namely AMASIL NA (Formic acid 94%), and astaxanthin, namely Lucantin Pink CWD (Astaxanthin 10%), on growth, survival and tolerance to *Vibrio parahaemolyticus* in Pacific white shrimp (*Litopenaeus vannamei*). The effect of these substances on the total number of bacteria, *Vibrio* spp. in the intestine of shrimp was also observed.

MATERIALS AND METHODS

Experiment 1: The effect of organic acids and astaxanthin on the growth and survival of *Litopenaeus vannamei* postlarvae

Experimental diets

The organic acid used in this study was AMASIL NA (Formic acid 94% and Sodium formate), while astaxanthin source was Lucantin Pink CWD (Astaxanthin 10%). Both organic acid and astaxanthin were provided by BASF The Chemical Company, Germany. Six experimental diets were formulated, namely: AMASIL NA at 0.3%, AMASIL NA at 0.6%, Lucantin Pink CWD 50 ppm, AMASIL NA at 0.3% + Lucantin Pink CWD 50 ppm, AMASIL NA at 0.6% + Lucantin Pink CWD 50 ppm, and the control group without AMASIL NA and Lucantin Pink CWD.

AMASIL NA and Lucantin Pink CWD were applied by dissolving their powders in distilled water, then spraying and mixing with commercial pellet feed.

Shrimp and experimental protocol

The experiments were carried out at the Aquaculture Business Research Center Laboratory, Faculty of Fisheries, Kasetsart University, Thailand. Postlarvae 9 (PL-9) of Pacific white shrimp were obtained from a hatchery in Chachoengsao Province, Thailand. After three days of acclimation, shrimp (now at PL-12 stage) were randomly distributed into 24 x 500-L fiberglass tanks (four replicate tanks per treatment). Each tank was stocked at a density of 75 shrimp.

Each treatment group was fed with the aforementioned diet four times daily for 60 days. Salinity throughout the experiment was maintained at 25 ppt, dissolved oxygen above 4 ppm, and water temperature at $29\pm1^{\circ}\text{C}$.

Growth and survival study

The average body weight and survival rate of shrimp were recorded after the 60 days experimental period.

Experiment 2: The effect of organic acids and astaxanthin on growth, survival, and intestinal bacteria of *Litopenaeus vannamei* challenged with *Vibrio parahaemolyticus*

Shrimp and experimental protocol

Shrimp in each tank in Experiment 1 were randomly distributed into 12 x 500-L fiberglass tanks (four replicate tanks per treatment). Each tank was stocked at a density of 30 shrimp. At the beginning of this experiment (Day 0), *Vibrio parahaemolyticus* isolated from shrimp which died from Early Mortality Syndrome (EMS) affected farms in Chanthaburi province were added into each tank. The final concentration of *V. parahaemolyticus* was 10^4 CFU/ml. Each treatment group received aforementioned diets four times daily for another 30 days. Salinity, dissolved oxygen, and water temperature were maintained as in Experiment 1.

Growth and survival study

The average weight gain and survival rate of shrimp from each treatment were recorded after 30 days of experimental period.

Intestinal bacterial study

Five shrimp individuals from each group were randomly selected for intestinal samples at the 10th, 20th, and 30th day. The individual intestines were homogenized and spread on TCBS (selective media for *Vibrio* spp. culture), then incubated at 37°C for 24 hours. Finally, all colonies of bacteria were counted and calculated according to CFU/g unit.

Statistical analysis

The data were analyzed using SPSS v 11.5 program. One way ANOVA and Duncan's New Multiple Range test were used to compare data among treatments. Differences were considered significant if $P<0.05$.

RESULTS

Experiment 1: The effect of organic acids and astaxanthin on the growth and survival of *Litopenaeus vannamei* postlarvae

After 60 days of dietary administration, shrimp fed with Lucantin Pink CWD 50 ppm showed the highest average body weight of 4.45 ± 0.45 g, followed by shrimp fed with AMASIL NA at 0.3% + Lucantin Pink CWD 50 ppm (4.38 ± 0.37 g), AMASIL NA at 0.6% + Lucantin Pink CWD 50 ppm (4.05 ± 0.21 g), and the control group (4.18 ± 0.05 g). The average weights of these groups were statistically different with that of the group fed with AMASIL NA at 0.3% (3.68 ± 0.49 g) and AMASIL NA at 0.6% (3.88 ± 0.22 g) (Table 1, Figure 1).

The average survival rate of shrimp fed with AMASIL NA at 0.6% + Lucantin Pink CWD 50 ppm was $82.33 \pm 8.32\%$ higher than those of the groups fed with AMASIL NA at 0.3% + Lucantin Pink CWD 50 ppm ($78.67 \pm 7.77\%$), Lucantin Pink CWD 50 ppm ($78.33 \pm 9.26\%$), AMASIL NA at 0.3% ($72.33 \pm 9.94\%$), and AMASIL NA at 0.6% ($67.67 \pm 14.29\%$). There were significant differences between the average survival rates of shrimp from these groups and the control group ($64.33 \pm 10.12\%$) (Table 1, Figure 2).

Table 1. The average body weight and average survival rate of Pacific white shrimp after 60 days of feeding with AMASIL® NA and Lucantin® Pink CWD

Treatment	Average body weight (g)	Average survival rate (%)
Control	4.18 ± 0.05 ^{abc}	64.33 ± 10.12 ^b
AMASIL® NA 0.3%	3.68 ± 0.49 ^c	72.33 ± 9.94 ^{ab}
AMASIL® NA 0.6%	3.88 ± 0.22 ^{bc}	67.67 ± 14.29 ^{ab}
AMASIL® NA 0.3%+ Lucantin® Pink CWD 50 ppm	4.38 ± 0.37 ^{ab}	78.67 ± 7.77 ^{ab}
AMASIL® NA 0.6%+ Lucantin® Pink CWD 50 ppm	4.05 ± 0.21 ^{abc}	82.33 ± 8.32 ^a
Lucantin® Pink CWD 50 ppm	4.45 ± 0.45 ^a	78.33 ± 9.26 ^{ab}

Data are presented as mean \pm standard deviation. Means in the same column with different superscript are significantly different from each other ($p<0.05$).

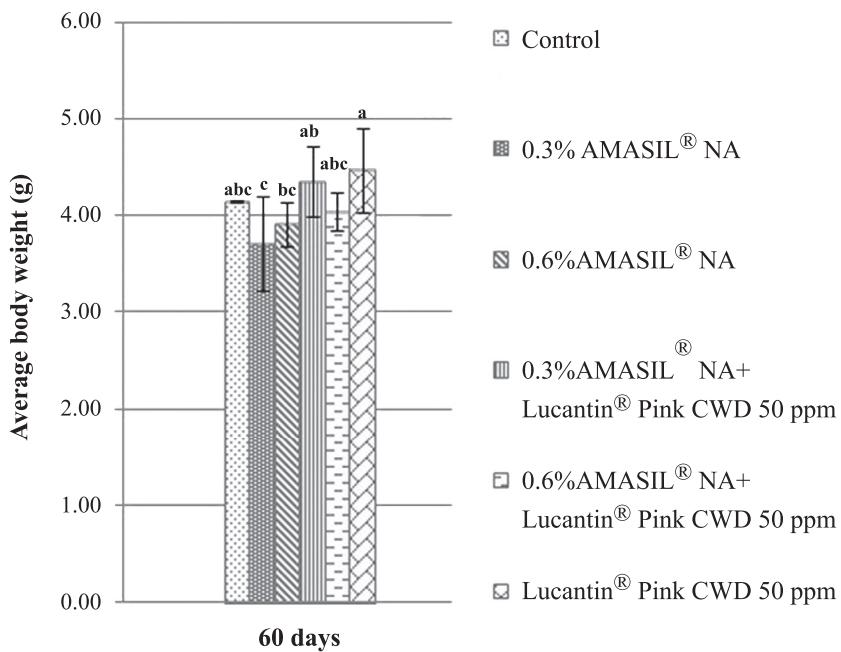


Figure 1. The average body weight of Pacific white shrimp after 60 days of feeding with AMASIL® NA and Lucantin® Pink CWD

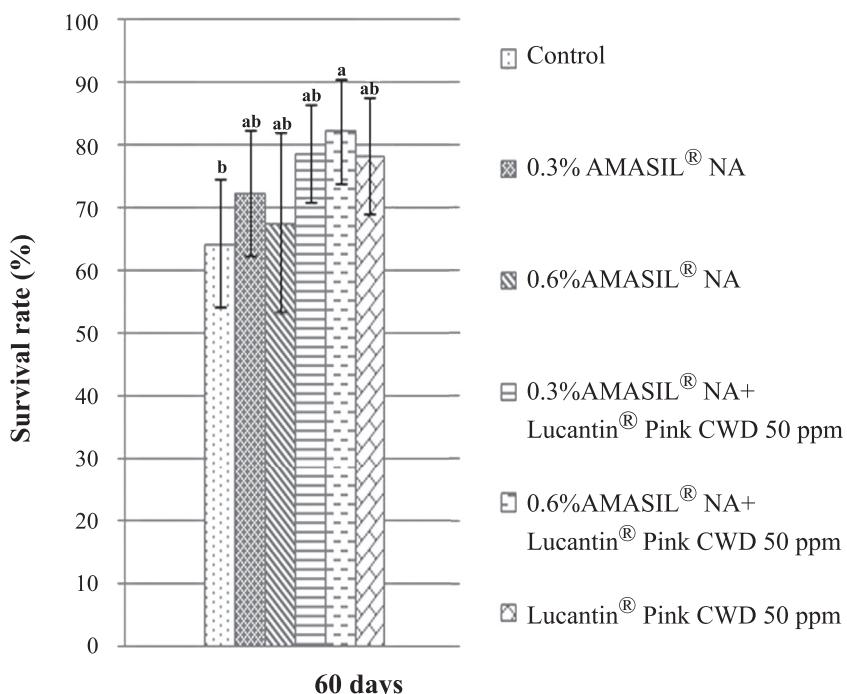


Figure 2. The average survival rate of Pacific white shrimp after 60 days of feeding with AMASIL® NA and Lucantin® Pink CWD

Experiment 2: The effect of organic acids and astaxanthin on growth, survival, and intestinal bacteria of *Litopenaeus vannamei* challenged with *Vibrio parahaemolyticus*

After the 30 day challenge trial, no significant difference in average weight gains of shrimp from the six experimental groups were observed (Table 2, Figure 3).

Table 2. The average body weight and average survival rate of Pacific white shrimp after challenging with *V. parahaemolyticus* 10^4 CFU.ml $^{-1}$ 30 days

Treatment	Average weight gain (g)	Average survival rate (%)
Control	2.73 ± 1.77^a	20.00 ± 17.32^c
AMASIL® NA 0.3%	2.13 ± 0.74^a	45.83 ± 16.78^b
AMASIL® NA 0.6%	1.82 ± 0.82^a	65.00 ± 5.77^a
AMASIL® NA 0.3%+ Lucantin® Pink CWD 50 ppm	2.97 ± 0.83^a	54.17 ± 5.09^{ab}
AMASIL® NA 0.6%+ Lucantin® Pink CWD 50 ppm	2.01 ± 0.69^a	67.50 ± 3.33^a
Lucantin® Pink CWD 50 ppm	2.39 ± 0.23^a	56.67 ± 17.11^{ab}

Data are presented as mean \pm standard deviation. Means in the same column with different superscript are significantly different from each other ($p < 0.05$).

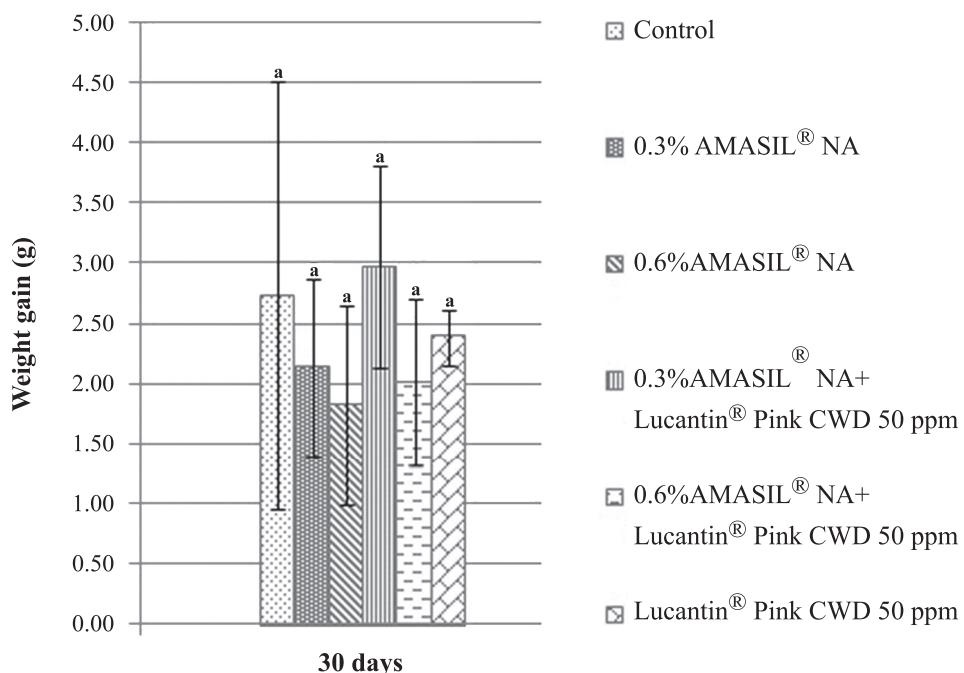


Figure 3. The average weight gain of Pacific white shrimp after challenging with *V. parahaemolyticus* (10^4 CFU.ml $^{-1}$) for 30 days

Shrimp fed AMASIL NA at 0.6% + Lucantin Pink CWD 50 ppm showed the highest average survival rate ($67.50 \pm 3.33\%$), followed by those fed with AMASIL NA at 0.6% ($65.00 \pm 5.77\%$), Lucantin Pink CWD 50 ppm ($56.67 \pm 17.11\%$), and AMASIL

NA at 0.3% + Lucantin Pink CWD 50 ppm ($54.17 \pm 5.09\%$). There were statistical differences between the average survival rates of these groups and those of the group fed with AMASIL NA at 0.3% ($45.83 \pm 16.78\%$) and the control group ($20.00 \pm 17.32\%$) (Table 2, Figure 4).

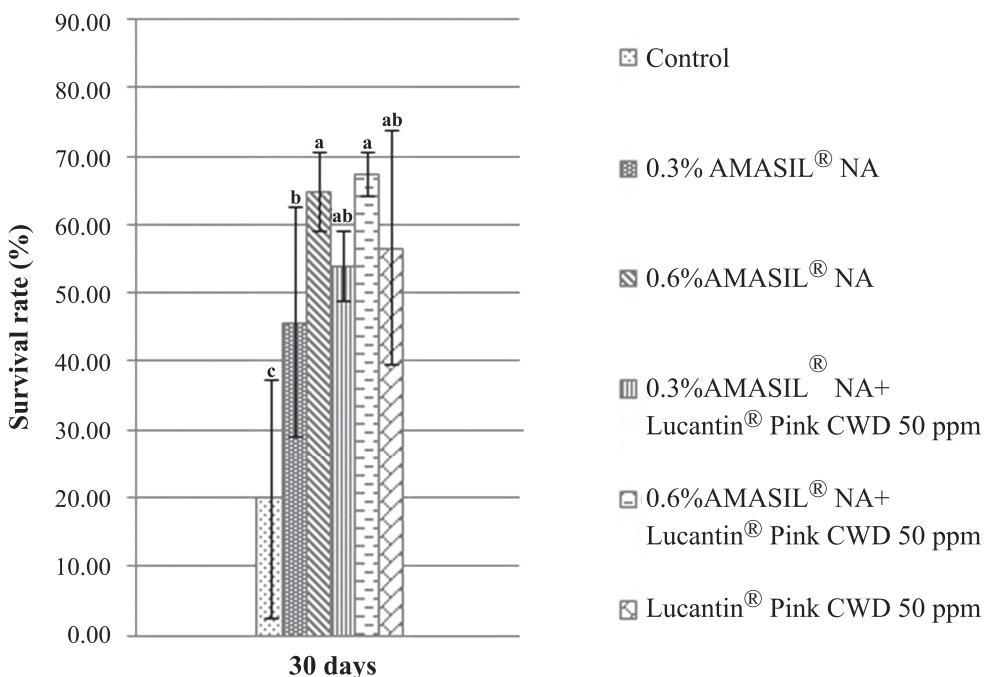


Figure 4. The average survival rate of Pacific white shrimp after challenging with *V. parahaemolyticus* (10^4 CFU.ml $^{-1}$) for 30 days

The average total number of *Vibrio* spp. in the intestines of the shrimp during the 30 days challenge with *V. parahaemolyticus* is shown in Table 3 and Figure 5. Shrimp in the group fed with AMASIL NA at 0.6% + Lucantin Pink CWD 50 ppm, AMASIL NA at 0.6%, AMASIL NA at 0.3% + Lucantin

Pink CWD 50 ppm, and AMASIL NA at 0.3%, had lower average total number of *Vibrio* spp. in their intestines throughout the feeding trial. The total number of *Vibrio* spp. in the intestines of shrimp in these groups was significantly lower than those in the group fed Lucantin Pink CWD and the control group.

Table 3. The average total number of *Vibrio* spp. (10^6 CFU/g) in the intestine of Pacific white shrimp after challenging with *V. parahaemolyticus* (10^4 CFU.ml $^{-1}$) for 30 days

Treatment	Total number of <i>Vibrio</i> spp. (10^6 CFU/g) in the intestine		
	10 days	20 days	30 days
Control	178.50±53.00 ^b	120.00±32.90 ^b	47.20±25.40 ^c
AMASIL® NA 0.3%	23.80±12.50 ^a	11.70±3.60 ^a	5.30±3.50 ^a
AMASIL® NA 0.6%	10.40±6.90 ^a	6.00±3.10 ^a	1.60±0.70 ^a
AMASIL® NA 0.3%+	26.70±11.50 ^a	10.80±2.90 ^a	5.40±2.20 ^a
Lucantin® Pink CWD 50 ppm			
AMASIL® NA 0.6%+	11.10±5.00 ^a	7.30±4.70 ^a	1.30±0.58 ^a
Lucantin® Pink CWD 50 ppm			
Lucantin® Pink CWD 50 ppm	168.30±100.50 ^b	119.00±74.20 ^b	27.80±11.30 ^b

Data are presented as mean ± standard deviation. Means in the same column with different superscript are significantly different from each other (p<0.05).

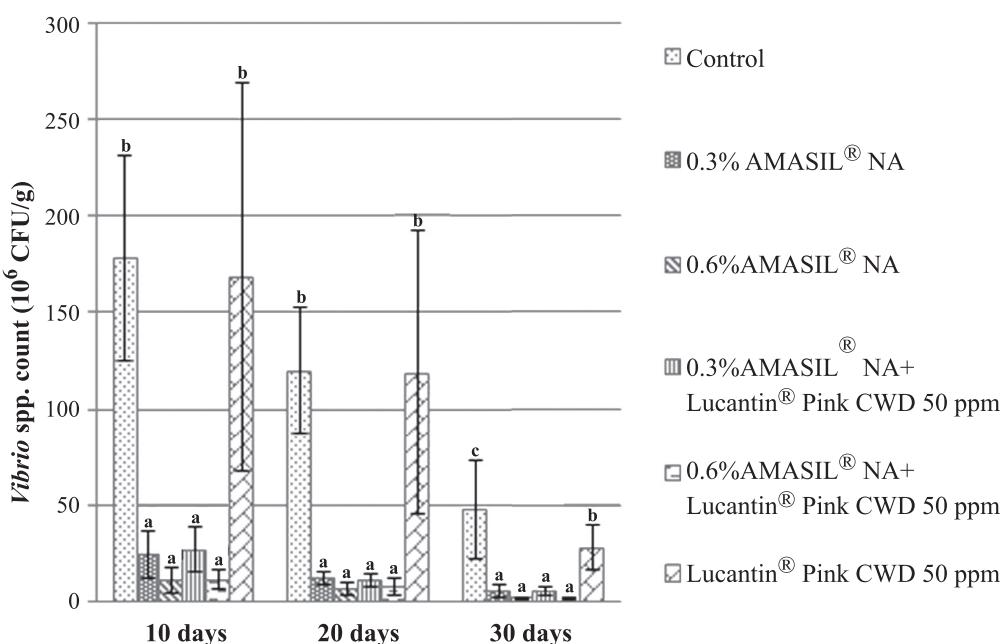


Figure 5. The average total number of *Vibrio* spp. (10^6 CFU/g) in the intestine of Pacific white shrimp after challenging with *V. parahaemolyticus* (10^4 CFU.ml $^{-1}$) for 30 days

DISCUSSION

Carotenoids are known to have a positive role in the metabolism of aquatic animals (Segner *et al.*, 1989) which could improve growth by enhancing nutrient utilization (Amar *et al.*, 2001). Many reports demonstrated that shrimp fed diets with supplemental astaxanthin had significantly improved growth performance and shortened moulting cycles at post larval stages (Thongrod *et al.*, 1995; Supamattaya *et al.*, 2005). However, the present study indicated that dietary astaxanthin had no effects on shrimp postlarval growth. The difference in the result could be associated with the dose of astaxanthin used in this study, which was 50 ppm lower than the 300 and 125 ppm recommended by Thongrod *et al.* (1995) and Supamattaya *et al.* (2005), respectively.

Organic acids are widely used as storage preservatives for food and to control pathogenic bacteria in feed ingredients (Defoirdt *et al.*, 2006). The mechanism of organic acids on growth improvement in a variety of fish species has already been reported (Baruah *et al.*, 2007; Hossain *et al.*, 2007). This is in contradiction with our results. Luckstadt (2008) revealed that the growth-promoting effects of dietary organic acids seem to depend on the animal species and type of organic acid tested. However, our result is similar to the recent study on Pacific white shrimp by Walla *et al.* (2012).

On the aspect of improving survival rates, Amar *et al.* (2001) reported that astaxanthin has beneficial effects on bio-defense mechanisms in rainbow trout. The fish fed with supplemental astaxanthin

exhibited better immune responses compared with the control group. Many reports also showed that dietary astaxanthin had a positive effect on shrimp survival (Yamada *et al.*, 1990; Chien and Jeng, 1992; Supamattaya *et al.*, 2005). These results are similar to the result in this study. Organic acids could improve gut health by lowering gastric pH or by diffusing into bacterial cells, then dissociating to release H⁺ cations to perturb cellular pH which results in bacteria cell death. Lowering the gut pH with the use of in-feed acidifiers has been observed in tilapia and *Labeo rohita* (Ng *et al.*, 2009; Baruah *et al.*, 2005). A recent study by Mine and Boopathy (2011) revealed the inhibitory effects of short-chain fatty acids, namely formic acid, acetic acid, propionic acid and butyric acid, on *Vibrio harveyi*. Among the four acids, formic acid showed the strongest inhibitory effect with the minimum inhibitory concentration (MIC) of 0.035%, followed by acetic acid, propionic acid and butyric acid. This observation also indicated that the major inhibitory mechanism seems to be the pH effect of organic acids. Walla *et al.* (2012) reported that oral administration of organic acids (fumaric acid and benzoic acid) could increase survival and reduce the bacteria *Vibrio* in the intestines of Pacific white shrimp, which is in agreement with the results our study.

CONCLUSION

Organic acids (AMASIL NA 0.3 and 0.6%) and astaxanthin (Lucantin Pink CWD 50 ppm) can enhance the survival rate of shrimp in laboratory conditions. Shrimp from the group fed with a combination of

AMASIL NA + Lucanthin Pink CWD, and the groups fed with either AMASIL NA only or Lucanthin Pink only, showed higher survival rates and more tolerance to *Vibrio parahaemolyticus* infection, compared to shrimp in the control group. This study also exhibited the benefit of using AMASIL NA to control the total number of *Vibrio* spp. in the intestine of shrimp. The total number of *Vibrio* spp. in the intestine of shrimp decreased with increasing AMASIL NA concentration in the feed.

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