

1 **Effects of Dietary Probiotic *Bacillus* sp. D2.2 and Sweet Potato**
2 **Extract on Growth Performance and Resistance to *Vibrio harveyi***
3 **in Pacific white shrimp, *Litopenaeus vannamei***
4

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13
14 **Abstract**

15
16 In this study, the effects of oral administration of probiotic *Bacillus* sp. D2.2 and prebiotic from sweet potato
17 extract on growth performance and resistance against *Vibrio harveyi* in Pacific Pacific white shrimp
18 (*Litopenaeus vannamei*) were investigated. During 32-day feeding experiment, 360 individuals of Pacific Pacific
19 white shrimp (PL15) with initial weight of 0.02 ± 0.002 g were fed with basal diet as control (A); supplemented
20 with 6% probiotic and 0% prebiotic (B); 6% probiotic and 2% prebiotic (C); 6% probiotic and 4% prebiotic (D).
21 After the feeding trial, weight gain (WG), average daily growth (ADG), feed conversion ratio (FCR), and
22 survival rate (SR) were assessed. Then, the best feeding treatment together with control were used for challenge
23 test with infectious *V. harveyi*. WG, ADG and FCR of the shrimp were significantly better in treatment D than
24 those of the shrimp in other treatments. Meanwhile, after the challenge test, survival rate and mean time to death
25 (MTD) of the shrimp fed the supplemented diet were not significantly different ($P>0.05$). Infection levels in
26 shrimp were evaluated using morphological scoring methods. Infection levels of *V. harveyi* in shrimp fed the diet
27 were lower compared with control.

28
29 **Keywords:** Probiotic *Bacillus* sp. D2.2; Prebiotic; Growth; Disease Resistance
30

31 **Introduction**

32
33 The demand for environment friendly in shrimpculture has increased mostly due to negative
34 side effects of antibiotic-resistant bacteria (Wright, 2010). Therefore, the use of antibiotics in aquafeed
35 has been restricted, such as in Europe (EC Regulation 1831/2003) and USA (U.S. Food and Drug
36 Administration, 2008). As an alternative, the uses of probiotics or prebiotics have heightened
37 attention. Probiotics are live microbial feed supplement that contributes to intestinal microbial balance
38 and maintains the organism's health (Socol et al., 2010). In recent years, several researches have
39 demonstrated that probiotics can enhance the disease resistance of shrimp by suppressing the
40

1 pathogens, enhancing immunity or improving water quality (Verschuere *et al.*, 2000). Thus, usage of
2 probiotics has been considered as one of the most promising preventive methods in aquaculture. Quite
3 a few microorganisms from the genus *Bacillus* have been used widely as putative probiotics.
4 Correspondingly, a number of researches have demonstrated that *Bacillus* can enhance the nutritional
5 and healthy benefits of shrimp (Rengpipat *et al.*, 1998; 2000; 2003; Li *et al.*, 2009). *Bacillus* sp. D2.2
6 that used in this experiment was non-pathogenic bacteria (Hardiyani *et al.*, 2016) and able to inhibit
7 the *in vitro* growth of *Vibrio harveyi* (Setyawan *et al.*, 2014). Meanwhile, prebiotics can increase
8 probiotics performance since prebiotics as non-digestible food ingredients benefit to selectively
9 stimulate the growth and/or activity of bacteria in the host's intestinal tract (Gibson *et al.*, 2004). In
10 addition, nutritional and health benefits of prebiotics oligosaccharides have been demonstrated in
11 shrimp (Li *et al.*, 2007; Zhou *et al.*, 2007; Li *et al.*, 2009).

12 Synbiotic is a combination of probiotics and prebiotics. It beneficially affects the host and
13 improves host welfare by improving the survival and colonization of live microbial dietary
14 supplements in the gastrointestinal tract, by selectively stimulating the growth and/or by activating the
15 metabolism of one or a limited number of health-promoting bacteria (Gibson and Roberfroid, 1995).
16 As the result of the negative effects such as the appearance of antibiotic-resistant pathogens and
17 concerns over the dispersal of antibiotic-resistant genes brought by using antibiotic, research on
18 replacement of antibiotic by synbiotic has been one of the hot topics on feed additive, which has
19 caused broad attention (Bengmark, 2005).

20 Previous studies have demonstrated that probiotics given in the form of synbiotics to the host
21 can enhance the survival of probiotics in gastrointestinal tract (Roberfroid, 2000; Bielecka *et al.*,
22 2002), and thus improving the quick reproducibility of probiotics *in vivo* and perform beneficial effects.
23 The aim of this study is to evaluate the effects of oral administration of probiotics (*Bacillus* sp. D2.2)
24 together with prebiotic of sweet potato extract on the growth and disease resistance of shrimp,
25 *Litopenaeus vannamei*.

Materials and Methods

Probiotic and prebiotic preparation

Bacillus sp. D2.2 was the probiotic bacteria used in the experiment that was isolated from traditional tiger shrimp farm in East Lampung (Setyawan *et al.*, 2014). The count of *Bacillus* sp. D2.2 was approximately 10^6 colony-forming unit (CFU) ml⁻¹. Probiotic bacteria was cultured in sea water complete-agar (SWC, 5 g bacto peptone, 1 g yeast extract, 3 ml glycerol, 15 g agar, 750 ml sea water, and 250 ml distilled water) and then transferred to SWC-broth (without agar) with rotary shaker at 140 rpm for 24 h at 30°C.

Production of prebiotic was started with production of sweet potato starch (Harpeni *et al.*, 2016). Extraction of oligosaccharide was using boiled water. As much as 5 g of sweet potato starch were mixed with 40 ml boiled water and continued to be stirred for 10 minutes in $85\pm 2^\circ\text{C}$ (Sukenda *et al.*, 2015). Two types of oligosaccharides, sucrose and raffinose, were analyzed by using High Performance Liquid Chromatography (HPLC) with the following results: sucrose 2.59% and raffinose 0.04%.

Experimental diet preparation

The basal diet was commercial pellets that contained approximately 30% crude protein and 5% crude lipid which were suitable for the growth of this shrimp. Four diets were used as experimental diets; Diet A (basal diet used as the control), Diet B (basal diet supplemented with 6% probiotic *Bacillus* sp. D2.2), Diet C (basal diet supplemented with 2% prebiotic and 6% probiotic *Bacillus* sp. D2.2), and Diet D (basal diet supplemented with 4% prebiotic and 6% probiotic *Bacillus* sp. D2.2). All supplements were thoroughly mixed with 2% egg yolk as binder (Sukenda *et al.*, 2015). Subsequently, the pellets were air dried at room temperature and stored in the plastic bags until used.

Culture condition

This study was conducted in the Aquaculture Laboratory, Department of Fisheries and Marine Sciences, Faculty of Agriculture, University of Lampung. The experiments used Pacific white shrimp

1 *L. vannamei* in post-larval stadia (PL) 15 which were obtained from a commercial hatchery in
2 Kalianda, South Lampung. Prior to the study, the post-larvae were acclimatized to laboratory
3 conditions for 5 days. Then the shrimp were weighed ($0.02 \text{ g} \pm 0.002$ mean initial weight), and
4 randomly distributed to four experimental groups, each of which had triplicate tanks 50x40x40 cm;
5 volume 40 litres). Each replication contained 30 shrimp, reared with the experimental diet for 32 days.
6 The experimental diets were provided at amounts equal to 8-10% body weight. Shrimp were fed to
7 apparent satiation three times daily. Water quality during the experiment was maintained by siphoning
8 out shrimp faeces and exchanging culture media at a rate of 10% daily. Water quality during the
9 experiment was kept at the following parameters: temperature 27-28 °C, salinity 29-32 ppt, dissolved
10 oxygen >3.5 mg/L, and pH 7.5-8.5.

11

12 **Growth trial**

13 After 32 days experiment, the total numbers of shrimp were counted and weighed. Weight
14 gain (WG), average daily growth (ADG), feed conversion ratio (FCR) as well as survival rate (SR)
15 were calculated using the following equations:

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$$\text{WG} = (\text{final weight} - \text{initial weight}) / \text{initial weight}$$

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$$\text{ADG} = \text{weight gain} / \text{days}$$

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$$\text{FCR} = \text{total dry feed intake (g)} / \text{wet weight gain (g)}$$

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$$\text{SR} = (\text{final number of shrimp} / \text{initial number of shrimp}) \times 100$$

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21 **Challenged test**

22 The challenge test aimed to study the performance of the best experimental diet during growth
23 trial in increasing *V. harveyi* resistance in Pacific white shrimp. The *Vibrio harveyi* isolate was
24 obtained from the Fish Health Laboratory, Center for Marine Aquaculture, Lampung. As many as 240

1 shrimp (PL 25) were distributed into 2 different treatments (basal diet as control and the best
2 experimental diet) and four replicate tanks. After receiving the experimental diet for 7 days, the shrimp
3 were infected with 10^6 colony-forming unit (CFU) ml^{-1} of *V. harveyi* by immersion. The shrimp were
4 then observed for seven days after the *V. harveyi* infection. The resistance parameters including SR,
5 relative percent survival/RPS (Khimmakthong *et al.*, 2011), mean time to death/MTD (Nitimulyo *et*
6 *al.*, 2005) were calculated while clinical signs and hepatopancreatic histological examination were
7 also observed.

8

9 **Data analysis**

10 The data obtained from growth trial were analyzed using one-way ANOVA followed by
11 LSD's multiple range test while relative percent survival (RPS) and mean time to death were analyzed
12 using one sample t-Test (IBM SPSS version 22). Clinical signs were scored based on the infection
13 levels of shrimp. Indicator of score 1 (light infection) was to lose appetite and balance, score 2 (mild
14 infection) was a reddened body and tail, score 3 (heavy infection) was gill damages, and score 4 (very
15 heavy infection) was hepatopancreatic damages until dead. Meanwhile, histological observation on the
16 hepatopancreatic damages such as necrosis, vacuolation and degeneration were also calculated.

17

Results

Growth performance

Shrimp fed with basal diet (Diet A) showed significantly lower average weight gain (0.50 ± 0.07) and ADG (15.7 ± 2.3) compared to other treatments. Significant differences in weight gain and ADG were observed among treatments. The higher percentage of prebiotic results in the higher weight gain and ADG. Similarly, average FCR among treatments are significantly different. The higher percentage of prebiotic results in the lower FCR. The FCR of shrimp fed with basal diet was 3.70 ± 0.40 , the highest FCR among other treatments. Survival of shrimp was high for all treatments ranging from 71 to 90%. No significant difference was found between diet A (control) and diet B (supplemented with 6% probiotic and 0% prebiotic); and also between diet C (supplemented with 6% probiotic and 2% prebiotic) and diet D (6% probiotic and 4% prebiotic) (Table 1). Based on the growth performance, the best experimental diet was diet D (supplemented with 6% probiotic and 4% prebiotic) and used in challenged test.

Resistance Parameters

Survival rate between basal diet and experimental diet (supplemented with 6% probiotic and 4% prebiotic) was not significantly different (87 and 95% respectively). RPS for the experimental diet was 53.48%. Shrimp fed with experimental diet had slower average time of death compared to shrimp fed with basal diet (75 ± 35.1 and 108 ± 27.3 respectively) (Figure 1). However, there was no significant difference between both diets.

Moreover, numbers of shrimp suffered from various infection levels of *V. harveyi* basically lower in group of shrimp fed with experimental diet. Mostly the shrimp suffered from mild infection (score 2) indicated with redness of tail (Figure 2).

After seven days of challenged test, more than 90% of degenerative hepatopancreas were prominent while around 36-52% of necrosis in hepatocytes and approximately 37-43% of vacuolation

1 were noticeable. Interestingly, hepatopancreas of shrimp fed with experimental diet had less damaged
2 than those fed with basal diet (Figure 3 and 4).

4 **Discussion**

5 The shrimp which were fed with experimental diets containing probiotic and or prebiotic
6 supplements showed better growth performance compared to those in the control group. After 32 days
7 of culture, weight gain and ADG of shrimp has improved while FCR has decreased. The highest
8 growth was measure in the Diet D treatment group. Other research has suggested the similar condition;
9 the dietary administration of synbiotics (application of probiotics together with prebiotics) can
10 influence the growth performance of shrimp (Merrieffield *et al.*, 2010). Administration bacteria via
11 dietary supplements in white shrimp may activate the shrimps' digestive enzyme (Lesmanawati,
12 2013). The improvement in digestive enzyme activities allows the host to digest and absorb more
13 nutrients (Cerezuela *et al.*, 2011). According to Ai *et al* (2011), gastrointestinal bacteria take a part in
14 the decomposition of nutrients, providing the host organism with physiologically active materials such
15 as enzymes, amino acids, and vitamins thus enhance food utilization and digestion. The increase in
16 growth and FCR as a result of dietary supplementation with synbiotics has been credited to
17 physiological and biological changes in the gastrointestinal environment (Daniels *et al*, 2010). Other
18 study indicates that the application of synbiotics allow for more efficient conversion of ingested food
19 into structural protein, with subsequent improved growth (Hai and Fotedar, 2009). Previous study
20 showed that prebiotic extracted from sweet potatoes could support the growth of probiotic bacteria
21 (Putra, 2010), such as *Bacillus* sp. D2.2 (Harpeni *et al.*, 2016). Ringo *et al.*, (2010) said that prebiotic
22 can selectively support the growth of specific species of bacteria in the digestive tract of shrimp.

23 Although survival rate of the shrimp fed with synbiotic supplementation was not significantly
24 increase compared with control diet, this experimental diet could protect the shrimp quite good with
25 RPS value 53.48%. Arisa (2011) reported that synbiotic may enhance resistance of white shrimp to *V.*

1 *harveyi*. Further study also revealed that combination of probiotic and prebiotic from sweet potato in
2 shrimp diets can significantly improve disease resistance presumably by enhancing immunity
3 (Nurhayati *et al.*, 2015). Mean time to death of the shrimp fed with experimental diet could be longer
4 than those consumed control diet. However, it seems that the experimental diet only protect shrimp
5 from bacterial attack not from development of bacterial infection. Therefore, mean time to death of the
6 experimental diet was not significantly different than of the control diet. Mild infection and mostly
7 degenerative hepatopancreas occurred in shrimp after challenged test. The experimental diet has
8 created less damage of hepatopancreas in shrimp.

9 This study showed that probiotic *Bacillus* sp. D2.2 with prebiotic from sweet potato in pacific
10 white shrimp (*Litopenaeus vannamei*) diets can significantly improve growth performance and could
11 protect the shrimp from bacterial infection by presumably enhancing immunity and modulating
12 microflora in the digestive tract of shrimp. The best dietary synbiotic in this study was basal diet
13 supplemented with 6% probiotic and 4% prebiotic.

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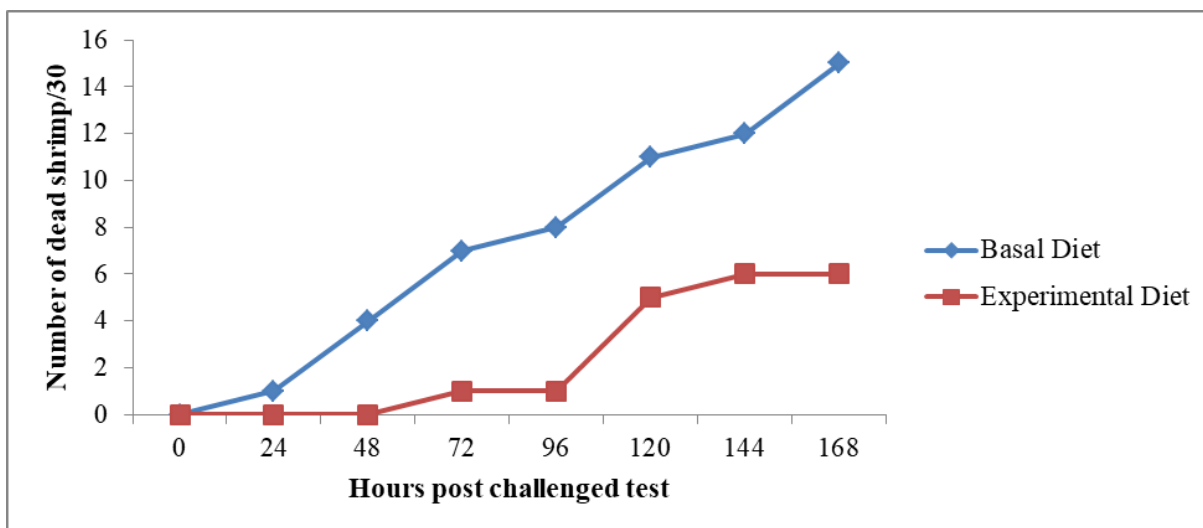
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1 Table 1. Growth and diet utilization of *L. vannamei* fed the experimental diets for 32 days.
 2 Means in a column with different letters were significantly different (P<0.05).

Treatments	Initial weight (g)	Final weight (g)	WG (g)	ADG (mg)	FCR (%)	SR ₃ (%)
A	0.02±0.002	0.52±0.07	0.50±0.07a	15.7±2.3a	3.70±0.40a	71.11±6.94a
B		0.83±0.07	0.81±0.07b	25.4±2.2b	2.09±0.20b	73.33±6.67ab
C		1.40±0.01	1.38±0.01c	43.1±0.4c	1.52±0.06c	83.33±3.33c
D		1.90±0.04	1.88±0.04d	58.6±1.3d	1.12±0.04d	90.00±3.33cd

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9 Figure 1. Mortality of shrimp after challenged test of two diets, i.e. basal diet and experimental diet
 10 (basal diet supplemented with 4% prebiotic and 6% probiotic *Bacillus* sp. D2.2)

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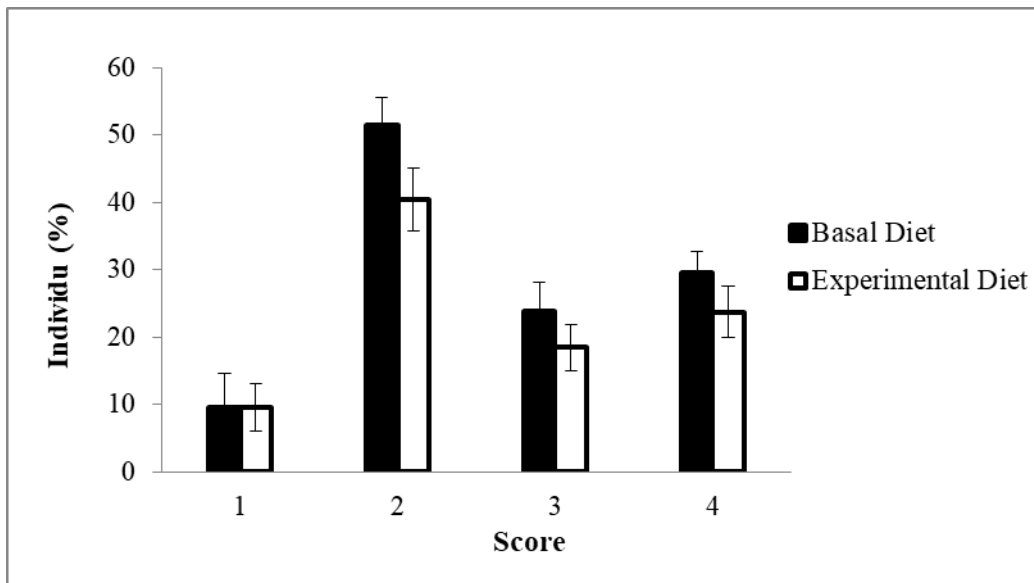


Figure 2. Scoring of clinical signs of shrimp after challenged test. Score 1 to 4 indicated light, mild, heavy and very heavy infections respectively.

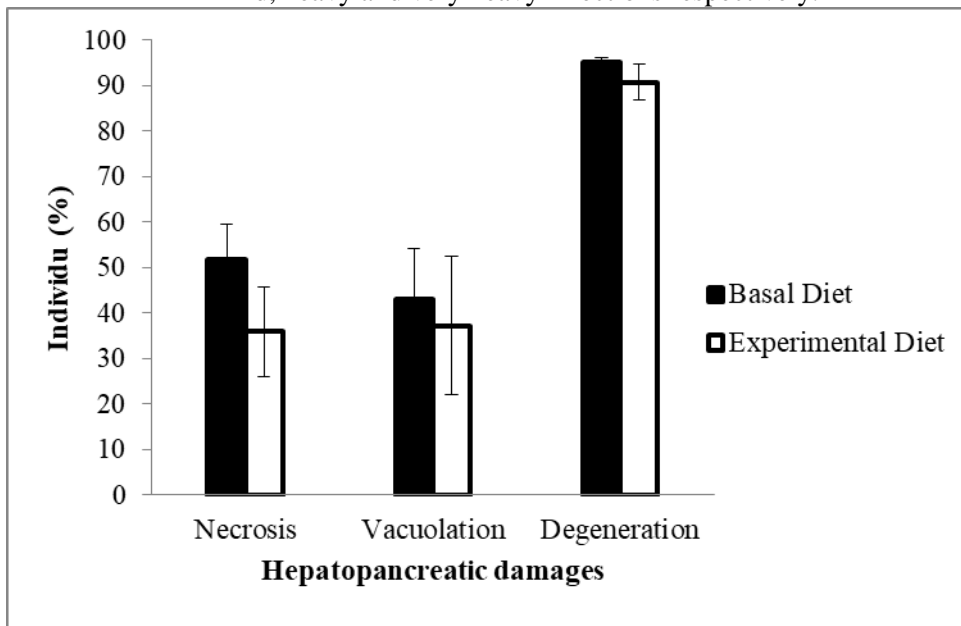


Figure 3. Number of shrimp suffered from hepatopancreatic damages after seven days challenged test towards two diets, i.e. basal diet and experimental diet (basal diet supplemented with 4% prebiotic and 6% probiotic *Bacillus* sp. D2.2)

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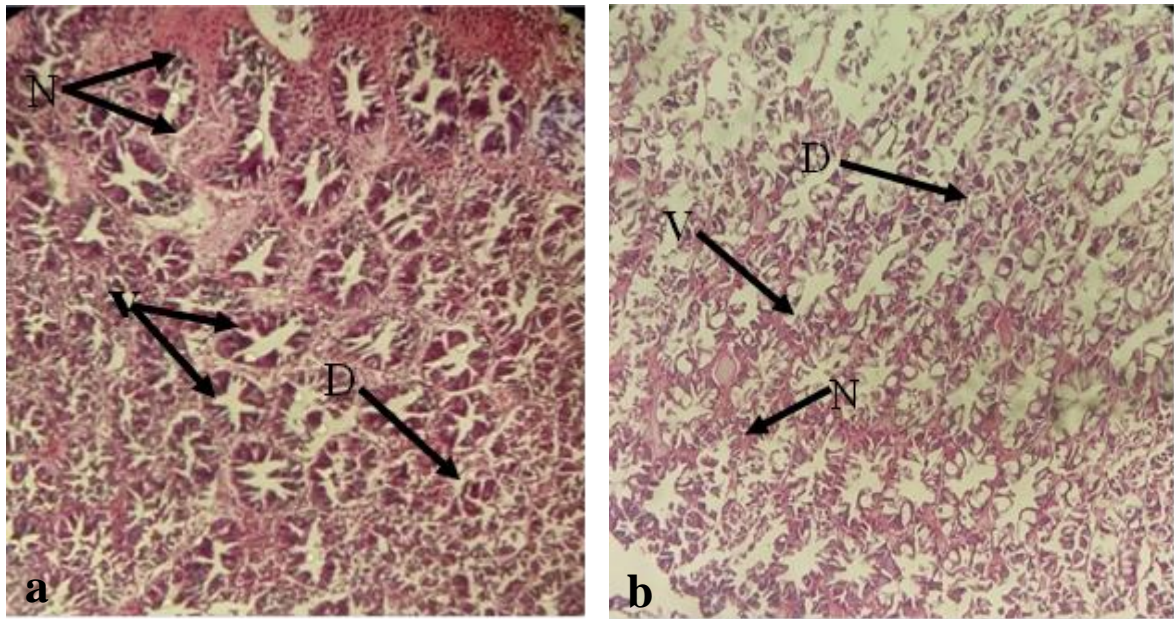


Figure 4. Comparative hepatopancreatic conditions between shrimp fed with basal diet (a) and shrimp fed with experimental diet (b) under light microscopy (400x). Hepatopancreatic damages including necrosis (N), vacuolation (V), and degenerative hepatopancreas (D).