Immune Profile of *Litopenaeus vannamei* in Monoculture and IMTA Ponds System

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Abstract

Integrated Multi Trophic Level (IMTA) is identified as the integrating culture of multi-species in one area. The effectiveness of space and feed is the main aspect from this point. The disease and health of shrimp management on shrimp production including immune boosters plays a critical aspect. The aim of this research is to determine and analyze the Litopenaeus vannamei immune system in different culture systems, namely polyculture (IMTA) and monoculture. Alginate was applied as supplementation diet by oral administration. The cellular and humoral immune parameters ie. Total hemolymph Count (THC), Phagocyte Activity (PA), Phagocyte Index (PI), Phenoloxidase activity (PO), and Superoxide dismutase activity (SOD) were assessed, monthly. A completely randomized design (CRD) with two treatments (IMTA and monoculture, and four replications in 500 m^2 pond area was conducted. In the third month, THC and PA of shrimp from the monoculture pond resulted in significant differences ($p<0.05$) compared to polyculture (IMTA). SOD activity from monoculture is significantly higher ($p<0.05$) at every sampling period. In PO enzyme activity of shrimps from monoculture pond produced higher results compared to IMTA ponds in the 2nd and 3rd months. It is concluded that L. vannamei monoculture ponds produced higher immune parameters compared to IMTA ponds. The food competition in terms of feed and space from IMTA ponds may affect this fact. Improving the feeding technique is recommended.

Keyword: IMTA, monoculture, immune, alginate

INTRODUCTION

Sustainable aquaculture or Integrated Multi-Trophic Aquaculture (IMTA) is defined as combining the multi-species in one area to get more benefits. In this system, the waste of one species will be consumed by other species, so, therefore the effectiveness, environmental, and economic friendly will be fulfilled. This will lead to an environmentally bio-remediation approach (Biswas *et al*., 2020; Khanjani *et al*., 2022; Loayza-Aguilar *et al*., 2023). Some research revealed that the survival rate of pooled fish and shrimp from IMTA system gained a higher total production (894.2 kg/ha-1695.7kg/ha) when compared to control (505,4kg/ha). There is about 3,48 - 1,6 times higher net profit and cost-benefit ratio from IMTA ponds. The IMTA treatment is a mix of (2000 ind/ha *Mugil cephalus*, 10000 ind/ha *Liza tade,* 30000 ind/ha *Penaeus monodon*, 2000 ind/ha *Crassostrea cuttackensis* and 200 kg/ha *Ipomoea aquatic*). On the other hand, the control is the mixture culture of 2000 ind/ha *Mugil cephalus*, 10000 ind/ha *Liza tade,* and 30000 ind/ha *Penaeus monodon*) (Biswas *et al*., 2020). Bartley, (2022) informed that in 2022, aquaculture from shrimp ponds provided 50% of the total fish from direct human consumption in the world, comes from the Asia-Pacific (Bartley, 2022).

Disease control eventually plays critical aspects in terms of every type of shrimp culture system, including IMTA (Integrated Multi-Trophic Aquaculture). The benefits of IMTA system are due to controlling disease through species interaction, which is supported by better water quality. Even though, some problems may appear (Khanjani *et al*., 2022; Zhang *et al*., 2022). Some pathogenic bacteria such as *Vibrio* spp., may be involved (Azhar & Yudiati, 2023; Hethesh *et al*., 2021), as well as pathogenic virus (White Spot Syndrome Virus/WSSV) (Yudiati *et al*., 2019). Alginate supplementation is a well-known compound for immune system boosters to combat pathogens and enhance disease resistance (Yudiati *et al*., 2019). This fact is in contrast with antibiotics which significantly correlates to antibiotic resistance in aquaculture (Aich *et al.*, 2018; Nadella *et al.*, 2021; Schar *et al.*, 2020).

The Indonesian brown seaweed, *Sargassum* sp. is a natural product that is rich in sodium alginate ie. 40,34% (Yudiati & Isnansetyo 2017). Alginate, extracted from *Sargassum* sp. cell wall

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contains polysaccharide for immunostimulant agent ((Jahromi *et al*., 2021; Yudiati *et al*. 2016) to enhance disease resistance (Azhar & Yudiati, 2023; Yudiati *et al.*, 2016). Alginat is composed by L-guluronate acid and D-mannuronate acid (Yudiati *et al.*, 2016; Yudiati *et al.*, 2019). In several studies, feed supplementation with alginate (*Sargassum* sp.) was evidently proven the improvement of shrimp resistence from pathogenic Vibrios namely *Vibrio alginolyticus* (Cheng *et al.*, 2005), *Vibrio harveyi* (Jiang *et al.*, 2017), *Vibrio parahaemolyticus* AHPND (Azhar & Yudiati, 2023) as well as WSSV (Chung *et al*. 2011; Yudiati *et al*. 2019). There is still a lack of information regarding the monoculture and this will even be more interesting to evaluate the IMTA pond system (polyculture) versus monoculture of *L. vannamei* with alginate supplementation in feed.

This present research is to evaluate the *Litopenaeus vannamei* immune system in IMTA (polyculture) pond system compared with monoculture towards alginate supplementation. The assessment covers the Total Haemocyte Count (THC), Phagocyte Activity (PA), Phagocyte Index (PI), Phenoloxidase activity (PO), and Superoxide dismustase activity (SOD).

MATERIALS AND METHODS

This research used field experiment with completely randomized design (CRD) using two treatments (poly n monoculture) and replicated four times (Fig. 1). Ponds were in 0,5 ha volume each. The polyculture ponds was the mixture of *L. vannamei* shrimp(10 ind/m^2), Miklfish (*Chanos chanos*) (5 ind/m^2), *Gracilaria* sp. (100 g/m^2), and *Anadara* sp. (50 ind/m^2). The monoculture ponds were reared solely with *L. vannamei*. *L. vannamei* PL 30 (0.5 $\pm$ 0.13 g) and *Chanos chanos* (1.0 $\pm$ 0.24 g) were purchased from farmer form East Java.

Alginate was puchased from private company and subjected to shrimp diet. The supplementation technique based on Azhar and Yudiati (2023); Yudiati *et al.* (2016); Yudiati *et al.* (2019). The alginate dose was 3.0 g/kg. Alginate was diluted with 100 mL aqua dest/kg feed. Mix well and added thoroughly with 1 kg shrimp feed (Feng Li®). Let it dry with oven (40 °C, 2 hrs). Based on data obtained, the water quality from shrimp pond media was fluctuated. Dissolved Oxygen was ranged from 4,61- 7,56 ppm, salinity was 15-30 ppt, pH was ranged from 7,31to 8,11 pH, and temperature was 29,2-33 ◦C. *L. vannamei* was reared for three months. The feed frequency was twice/day at 05.00 and 17.00. Water exchange was 10 % from total volume daily. The haemolymph sampling to evaluate the immune parameters was taken from four shrimps from each pond, and was cunducted every month.

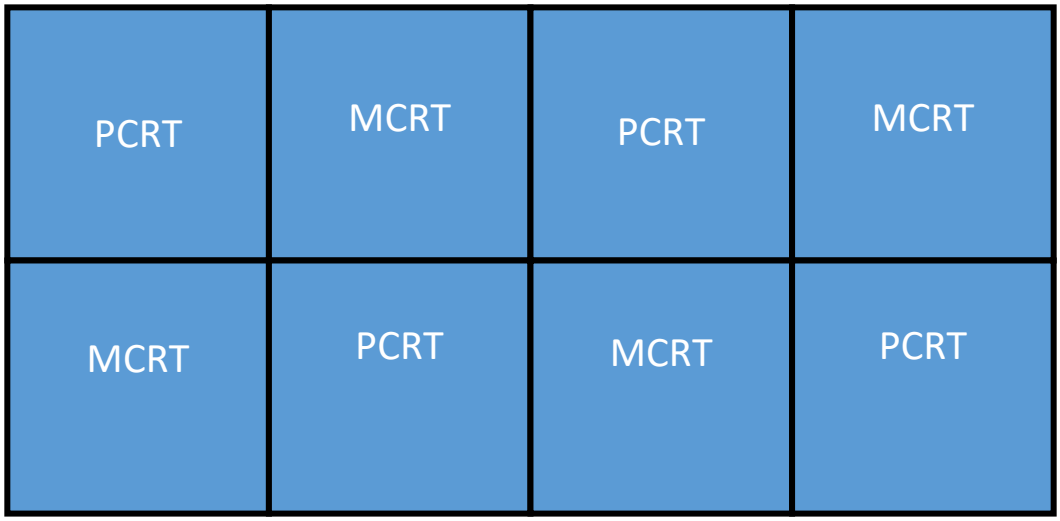

Figure 1. The lay out of Poly (IMTA) and Monoculture of *L. vannamei* ponds.
Note : PCTR= Polyculture; MCTR = Monoculture

Total haemocyte count, phagocyte activity, and phagocyte index

Total hemolymph count (THC), phagocyte activity (PA), and phagocyte index (PI) were conducted by the previously described procedures (Azhar & Yudiati, 2023; Yudiati *et al.*, 2016; Yudiati *et al.*, 2019). Four Shrimps from each IMTA (polyculture) and monoculture ponds were randomly sampling. Twenty µL of haemolymph was diluted with 80 µL NaCl 0,8% in aqua dest. Haemocyte was calculated under light microscope in a haemocytometer.

To determine the PA and PI, haemolymph and Phosphate buffer saline was mixed 1:3 (v:v)in 1.5 mL microtube. The 10^{8} sel/mL *Bacillus* sp. formalin killed was then added to the haemolymph 1:1(v:v). This followed by taking up 5 µL of this mixture and then smeared in glass slides, and let it dry. Fixation with 95% ethanol was then applied. Giemsa 10% was used as colouring agent. Finally, slides were rinsed with water and dried in ambient temperature. Photograph was taken and assessment was done under light microscope.

Phenoloxidase (PO) and Super oxide dismutase (SOD) activity assay

PO activity was measured spectrophotometrically by recording dopachrome formation from L-dihydroxyphenylalanine (L-DOPA) (C. H. Liu *et al.*, 2004). SOD activity was measured spectrophotometrically by measuring riboflavin production with nitroblue tetrazolium (NBT) (Beauchamp and Fridovich 1971).

Statistical analysis

The data was then statistically analyzed using R-Studio to determine the differences between treatments. The data was tested by one-way analysis of variance (ANOVA) to determine whether the treatment had a significant effect ($p < 0.05$) or not. To determine the differences in the THC, PI, PA, PO and SOD among treatments, LSD was tested.

RESULTS AND DISCUSSION

Total Haemocyte Counts, Phagocytic Activity and Phagocytic Index

The shrimp haemolymph, similar to vertebrate blood is referred to the circular haemocyte from shrimp coelom. Haemolymph plays an important role in every physiological function, such as immune response (Coates & Costa-Paiva, 2020; Fredrick & Ravichandran, 2012; Gianazza *et al*., 2021). Shrimp is not completed with adaptive immune system, so, therefore shrimp is absolutely rely on their innate immune system which divided into cellular and humoral response (Fredrick & Ravichandran, 2012). The invertebrate immune response depends on innate instead of adaptive effector (Gianazza *et al*., 2021). Shrimp haemolymph plays a significant role in immune response. Haemolymph, which contains haemocyte involves in recognizing and destroying pathogens. Haemolymph is also invloved with wound recovery and tissue recovery. Hemolymph also consist of several type of proteins interrelated with immune and antimicrobial peptide sinergizing the protection from infection (Kumar *et al.*, 2023).

The Total hemolymph count (THC), phagocyte activity (PA), and phagocyte index (PI) is showed in Figure 2. At 3rd month, the Total hemolymph count (THC) of shrimps from monoculture pond system has significant (0.05) results compared to polyculture ponds (Fig. 2a), and this data is in line with PA data. On the other hand, in terms of THC and PA parameters, there are no sinificantly effect ($p>0.05$) at 1st and 2nd months. Different from PI, there is no significantly differences between shrimps from poly and monoculture ponds (Fig. 2 c). Based on previous research before, alginate consistently have the ability to enhance THC (Azhar & Yudiati, 2023; Yudiati *et al.*, 2016; Yudiati *et al.*, 2019) and PA (Yudiati *et al.*, 2016; Yudiati *et al.*, 2019). In contrary, another researcher (C.-H. Liu *et al*., 2006) reported, in *Penaeus monodon* culture, the supplementation of sodium alginate has no significant effect. Due the cell to cell communication, phagocytic activity is also related to total hemocyte count, reactive oxygen species (ROS), and SOD. In terms of combating the pathogen

such as Vibrio spp., phagocytosis in *L. vannamei* is associated with producing highly microbiocidal ROS (Muñoz *et al.*, 2000).

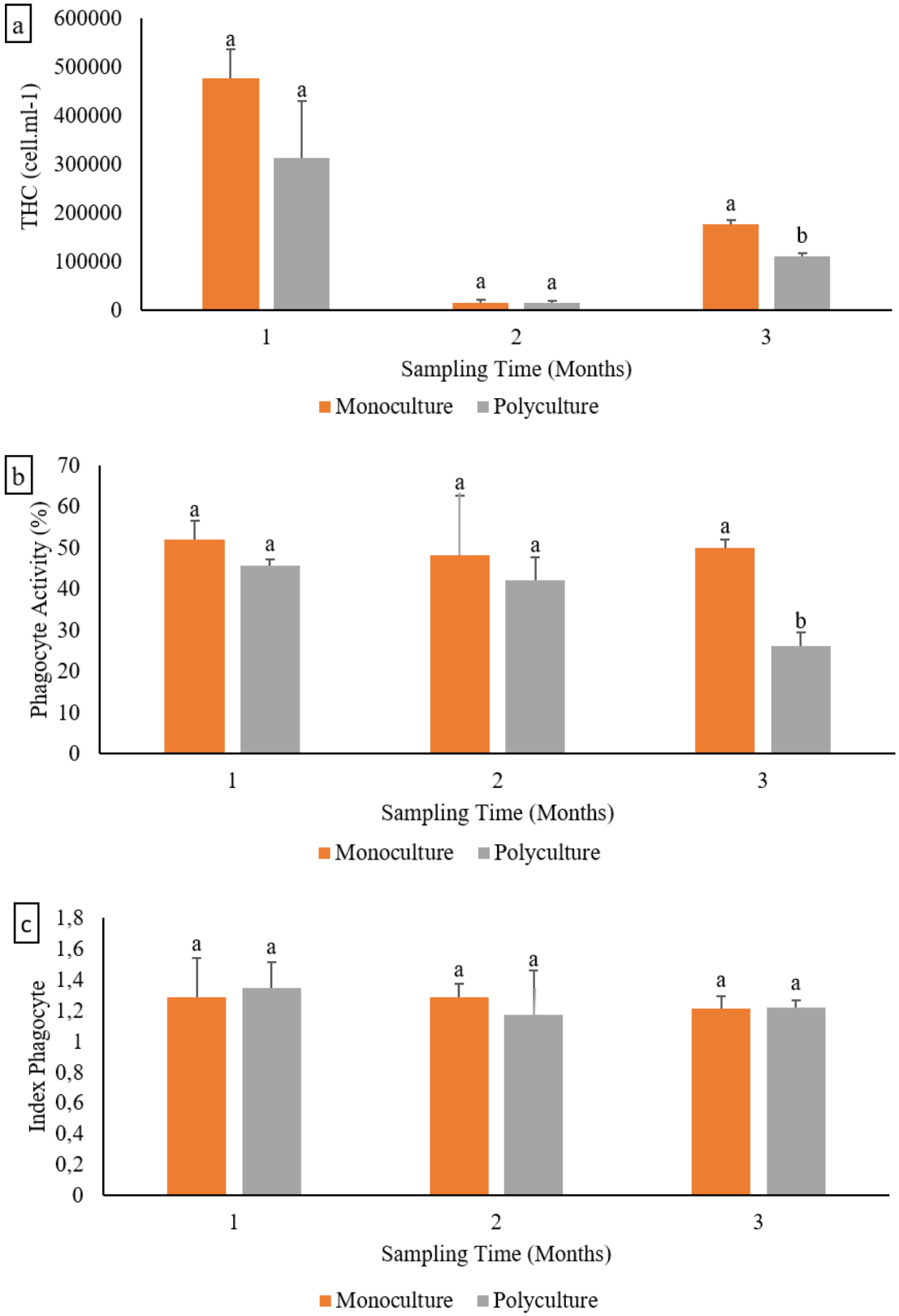

Figure 2. Total hemolymph count (a), phagocyte activity (b), and phagocyte index (c)of shrimps from poly and monoculture ponds

Super oxide dismutase (SOD) activity assay

This microbiocidal metabolite is converted by the antioxidant superoxide dismutase (SOD) into hydrogen peroxide, which can freely pass through membranes. Catalase and glutathione peroxidase can eliminate hydrogen peroxide from cells. Although these microbiocidal agents are produced in the phagocytic vacuoles, a critical amount enters the extravascular and extracellular environment and may cause cell damage (Warner, 1994). The Super oxide dismutase (SOD) enzyme activity in IMTA(Polyculture) and monoculture is depicted in Fig. 3. SOD from shrimp monoculture ponds is higher significantly (0,05) when compared from IMTA (Polyculture) in every sampling month. Research from Cheng *et al*. (2005) shows that supplementation of 2 g/kg sodium alginate of *L. vannamei* juveniles was able to arise the respiratory burst, and SOD activity. Different from *L. vannamei*, it is reported that sodium alginate is not impacted to the SOD activity of *P. monodon* (C.-H. Liu *et al.*, 2006).

Phenoloxidase (PO) activity assay

Prophenoloxidase system and its gene expression have an important role in shrimp immune responses towards pathogens. The activation of the proPO system by the specific recognition of microorganisms (PAMPs) such as lipopolysaccharides (LPS) to pattern-recognition proteins (PRPs) of the host initiates a serine proteinase cascade, which results in the cleavage of the inactive proPO to the active PO. This then follows by some mechanisms to produce melanine and toxic reactive intermediates against invading pathogens (Amparyup *et al.*, 2013). The Phenoloxidase (PO) activity in IMTA(Polyculture) and monoculture is presented in Figure 4. In the 1st month, PO activity of shrimps from monoculture is not significantly different ($p>0.05$) compared to polyculture. According to the rearing time, in 2nd and 3rd months, PO activity of shrimps from monoculture ponds is significantly higher compared to polyculture shrimps. Researchers noticed that *L. vannamei* juveniles fed diets containing sodium alginate 2.0 g/kg could enhance the immune ability of phenoloxidase activity (Cheng *et al.*, 2005). Consistently, there is no significantly differences in PO, in terms of using sodium alginate feed supplementation for *P. monodon* (C.-H. Liu *et al.*, 2006).

Biswas *et al*. (2020) reported IMTA provides the more effective production, environmentally and economically and yielded in higher total production ie. 894.2 kg/ha-1695.7kg/ha), when compared to control (505,4kg/ha). It is clearly shown that there is an increment (3.48 and 1.6 times) in terms of net profit and cost-benefit ratio. Based on this present research, there is no stimulation in terms of

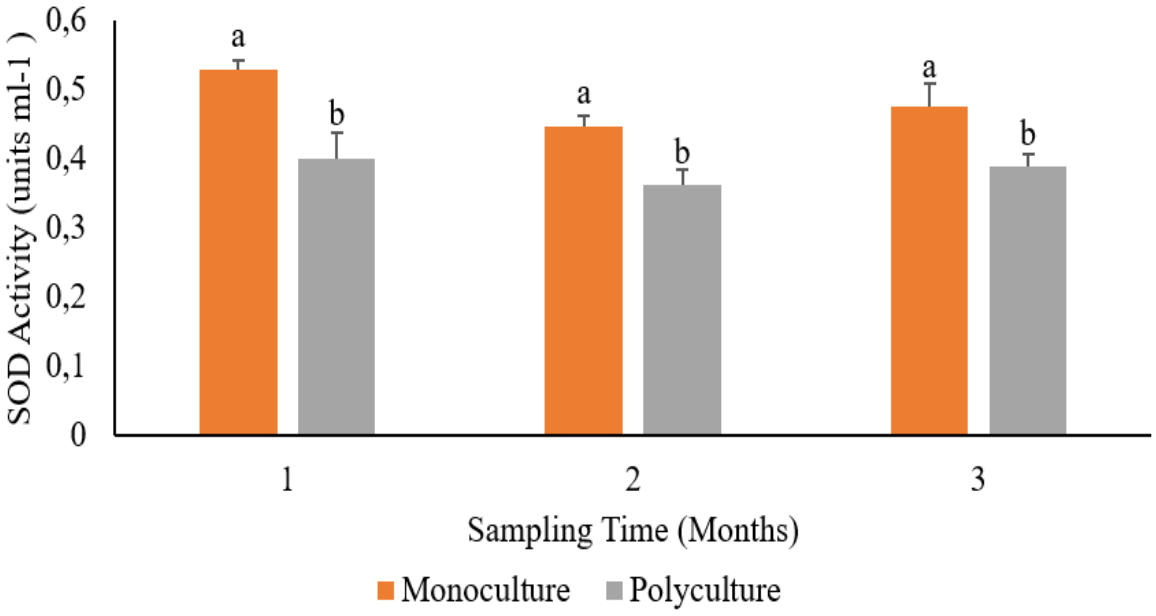

Figure 3. Super oxide dismutase (SOD) activity of shrimps from poly and monoculture ponds.

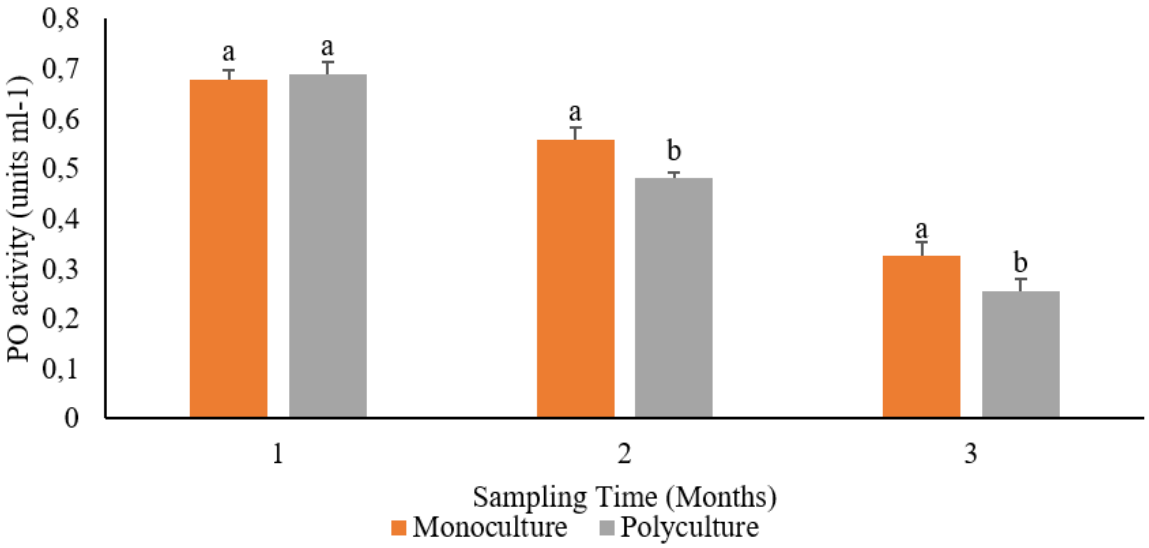

Figure 4. Phenoloxidase (PO) activity of shrimps from poly and monoculture ponds

immune responses. This due to the fact of feed competition between *L. vannamei* and *Chanos chanos*. *Chanos chanos* is a free-swimming fish. Though they live in the water column, shrimp and fish have the different feeding behaviour. Shrimps will bring feed, bite and ingest in a longer time than milkfish. Milkfish was a strong competitor, and consumed supplemented shrimp feed, in higher amount rather than shrimp (data not shown). It is suggested to apply the correct feed technical methods.

CONCLUSION

The immune parameters (THC, PA, PO, and SOD) of shrimp from monoculture ponds is higher than polyculture (IMTA). This due to the feed competition of *Litopenaeus vannamei* and *Chanos chanos* from polyculture ponds.

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