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Effect of *Lactobacillus salivarius* Dietary Supplementation on the Antioxidant Biomarkers of the Freshwater Shrimp *Macrobrachium rosenbergii*

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ABSTRACT

The shrimp aquaculture is one of the most important livelihoods in the Philippines. Although there is an observed boost in the production of shrimps in the Philippines, there are remaining problems that are needed to be resolved. Bacterial and viral infections in shrimps have significantly affected its mortality, resulting in lower production and yield. In order to prevent or lessen this effect, various researches have been made to increase the resistance of shrimps against infections. In this study, a probiotic microorganism, *Lactobacillus salivarius* was used as a dietary supplementation for *Macrobrachium rosenbergii* and see its influence on the antioxidant biomarkers and temperature stress tolerance of the shrimp. Three concentrations of *L. salivarius* ($1.0x10^7$, $1.0x10^8$, and $1.0x10^9$ cells/g) were initially tested for its effect towards the oxidative stress tolerance and the oxygen consumption of the shrimp. The study involved the use of various antioxidant biomarkers, including total glutathione (tGSH), catalase enzyme activity, and lipid peroxidation. Results showed that $1.0x10^8$ cell/g of *L. salivarius* was the most effective concentration of probiotic micro organism per weight of feeds in improving the metabolic rate of the shrimp. Oxidative stress tolerance levels were observed to be increasing in terms of the catalase reaction (12.46 and 21.74 nmole/min) and lipid peroxidation ($1.4x10^{-7}$ and $1.49x10^{-7}$ nmole MDA/mg protein) on both hepatopancreas and muscles tissues when the shrimp was fed with supplemented feeds. Also, high oxidant stress biomarkers were more evident on the hepatopancreas than the muscles.

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1. INTRODUCTION

Aquaculture is one of the primary food-producing sector around the world and continuously growing industry (Iyapparaj et al. 2013). Aquaculture in the Philippines has been practiced for a long time, and it involves different farming practices depending on the ecosystem (FAO 2005). Main products of aquaculture in the Philippines are tilapia, milkfish, seaweeds, carp, shrimp, oyster, and mussel (FAO 2005). Shrimp aquaculture is practiced in the Philippines primarily as a source of livelihood and for commercial purposes and attracts more attention because of its economic interest. Freshwater shrimps in the country were first given focus in the year 1914 by Cowles, wherein he identified that freshwater prawn was one of the essential fisheries during that time (Rosario and Tayamen, 2004). Shrimp aquaculture in the country became very high from 1989 to 1994 and generated 80% of total production and 20% in marine capture fishery (Salayo 2000).

Among the widely distributed shrimp species is *Macrobrachium rosenbergii*, locally called as "ulang." It is one of the most desirable candidates for freshwater aquaculture (Cuvin-Aralar et al. 2007) because it is fast growing and can quickly adapt in freshwater and low brackish water conditions (Seenivasan et al. 2012).

The resistance of *M. rosenbergii* to most of the viral diseases, tolerance in a wide range of environmental factors and most high market demand has increased the interest in local fisheries (Dash et al. 2014). There has been extensive research on M. rosenbergii, ranging from fisheries, aquaculture, taxonomy, morphology, development, anatomy, physiology, biochemistry, ecology to even social behavior (Wowor and Ng 2007).

Though shrimp aquaculture has been established in the country, there are still numbers of challenges that are being faced by the shrimp farmers that affect the growth and yield of shrimps. Some of which

include fluctuations in environmental conditions and the acquisition of bacterial and viral diseases.

The culture of freshwater prawn in the Philippines is intended to produce more commodities and option in the aquaculture industry (Rosario and Tayamen 2004). Freshwater prawns grow in either freshwater or brackish water environment, in addition to its many advantages for commercial culturing such as it can grow in captivity, relatively large, and has a rapid growth rate (Rosario and Tayamen 2004).

Various studies have been conducted in order to increase the rate of production of commercially available shrimps to compensate for the high market demands. One of the methods of attaining such an objective is the introduction of supplements to the shrimp diet. Probiotic supplementation in the aquaculture industry is very beneficial to shrimp metabolism in improving its feed digestion and absorption (Liu et al. 2010; Venkat et al. 2004).

Probiotic supplements can inhibit pathogenic microorganisms, induce the release of growth-promoting factors, and boost the immune response (Seenivasan et al. 2012; Verschuere et al. 2000; Venkat et al. 2004). Among the most widely used probiotic sources are the lactic acid bacteria such as Lactobacillus and Bacillus species that were shown to have a good response towards the improvement of the shrimp's immune response against viral infections (Chiu et al. 2007; Liu et al. 2010; Zokaeifar et al. 2012).

Lactobacillus acidophilus is usually found on the duodenal mucosa of animals and even isolated in human breast milk. This organism is probiotic bacteria if it possesses good growth performance in the intestinal microflora, can survive to an acidic environment, have bile hydrolase activity, and have antibacterial activity against gastrointestinal pathogenic bacteria (Chen et al. 2017).

Also, the environmental conditions such as temperature and salinity rate are essentials in determining the physiological responses and survival of *M. rosenbergii*. The shrimp distribution within estuarine systems is greatly influenced by temperature. With this in mind, the study aimed to determine the influence of *Lactobacillus salivarius* supplement on the antioxidant biomarkers and temperature stress tolerance of *M. rosenbergii*. We hypothesize that with varying environmental conditions such as temperature the metabolic rate of the *M. rosenbergii* is expected to fluctuate at certain points.

2. MATERIALS AND METHOD

2.1. Sample Collection

A total of 50 pieces juvenile *M. rosenbergii* with 5 cm to 7 cm was acquired from the Southeast Asian Fisheries Development Center (SEAFDEC), Binangonan Freshwater Station in Binangonan, Rizal. Before experimentation, the shrimps were acclimatized for two weeks in a full plastic container with proper aeration system at 25°C.

2.2 Bacterial Culture, Feed Preparation, and Feed Management

Lactobacillus salivarius was obtained from the UST Collection of Microbial Strains of the University of Santo Tomas, España, Manila, Philippines. The bacteria were revived and cultured in de Man, Rogosa, Shrape (MRS) agar (Chen et al. 2012). After 24 hrs, a loopful of *L. salivarius* was transferred in a 20 mL MRS broth. After 24 hrs, the bacterial suspension was centrifuged for 10 minutes at 10,000 rpm. The supernatant was discarded, and the bacteria were suspended in a sterile phosphate buffer (pH 7.5). The bacterial density was adjusted to $1.0x10^7$, $1.0x10^8$, and $1.0x10^9$ cells/mL and was added with 10 g of sterile powdered shrimp pellets containing 1% starch as a binder (Alambra et al. 2012; Maningas et al. 2013) for each of bacterial density. After which, the feeds were placed in a clean plastic container and dried at 37°C for 24 hrs. There were four groups of shrimps used in the experiment. The control group was fed with standard commercial feeds while the other shrimp groups were fed with commercial feeds with *L. salivarius* at $1.0x10^7$ (LS7), $1.0x10^8$ (LS8), and $1.0x10^9$ (LS9) cell/g of feeds concentration. The shrimps were fed with 1 g of feed once a day for three weeks, and the sampling was done once a week.

2.3 Water Quality Assessment and Metabolic Rate Determination

Water quality parameters, including temperature, dissolved oxygen, and pH were measured using Xplorer GLX Water Quality Studies bundle. These parameters were used to determine the metabolic respiration of the shrimp in its response to the environmental stress applied (Liu et al. 2010). Metabolic rate based on oxygen consumption was measured through the dissolved oxygen in the set-up. The set-up for the metabolic rate determination was prepared by immersing the Xplorer GLX water quality studies bundle in a closed set-up followed by measuring the initial dissolved oxygen. The shrimp was then put on the closed set-up, followed by the stress induc-

tion. The oxygen consumption was measured by the difference of initial reading of dissolved oxygen and the final reading of dissolved oxygen. Metabolic rate based on oxygen consumption of the shrimp was expressed as oxygen consumption in mL per weight of the shrimp sample in one hour of stress induction.

In addition to that, ammonia (NH_3/NH_4^+) and phosphate (PO_4^{-3}) contents were both measured using commercially available kits (API Fishcare Brands, USA). Analyses of water quality were done in triplicates.

2.4 Sample Preparation and Antioxidant Biomarkers Bioassays

Hepato-pancreas and muscles from the shrimp were collected and homogenized in an ice-cold buffer containing 50mM KH_2PO_4/K_2HPO_4 (pH 7.5) using IKA Automated Homogenizer (IKA, Malaysia). Bradford assay was performed to quantify the protein present both in the hepato-pancreas and muscle of each shrimp (Bradford 1976). Analyses of samples for the assessment of antioxidant biomarkers were done in triplicates.

Enzymatic catalase test was performed based on the protocol described by Vinagre et al. (2014) with some modifications. Briefly, a mixture of 384 µL phosphate buffer, 197 µL hydrogen peroxide (30%), and 19.7 µL tissue homogenates were prepared. Then, the absorbance was read at 240nm using Corona Microplate reader SH-1000 (Hitachi, Japan).

The total glutathione content was measured based on the reaction of reduced GSH with 5-5'Dithio-bis-2-nitrobenzoic acid (DTNB) (Tu et al. 2012). Briefly, 100 µL of tissue homogenate was added with 100µL of 5% sulfosalicylic acid and was incubated for one hour at 4°C. Then, the tubes were centrifuged for 15 minutes at 1,200 X g, and 10 µL of the supernatant was added with 270 µL phosphate buffer (pH 7.5) and 20 µL DTNB (Sigma-Aldrich, Germany). The absorbance was then read at 412 nm using Corona Microplate reader SH-1000 (Hitachi, Japan).

Lipid peroxidation assay through the Thiobarbituric Acid Reactive Substances (TBARS) was done using a TBARS assay kit (HiMedia) (Mensah et al. 2012). Malondialdehyde standards were prepared, starting from 125 µM. A 100 µL of lysed cells was mixed with 100 µL of SDS solution. The solution was vigorously mixed while adding 4mL of Thiobarbituric acid solution and placed on a boiling water bath for 1 hour. After 1 hour incubation, the solution was removed and immediately placed in an ice-cold water bath for 10 minutes. A sample of each treatment was transferred into a centrifuge tube and run at 1,600 X g for 10 minutes. The solution was kept at room temperature for 30 minutes to become stable. Samples were then loaded in a 96-well plate and read at 540 nm.

Glycogen in hepatopancreas was extracted in 5% trichloroacetic acid and homogenized for 2 minutes. The sample was centrifuged for 6 minutes at 7,000 rpm, and the supernatant was collected. Two hundred microliter of the supernatant was transferred in a tube containing 95% ethanol and was incubated for 3 hours at 37°C. Then, the tubes were centrifuged again at 7,000 rpm for 15 minutes, and the supernatants were discarded. The glycogen pellets were dissolved in 0.5 mL boiling water, 1 mL of concentrated sulfuric acid, and 200 µL 5% phenol (Rosas et al. 2000). The solution was then transferred into a microplate, in triplicate, and absorbance was read at 490 nm.

2.5 Water Temperature Stress Test on Shrimp fed with Dietary Supplementation

For the experiment, the protocol of Tu et al. (2012) and Vinagre et al. (2014) were followed with some modifications. The shrimps were subjected into two temperatures, 20°C and 35°C. Temperatures such as 20°C and 35°C were used as a stressor for the shrimp before metabolic rate determination and antioxidant biomarkers. Shrimps were divided into four groups:

CF20 - shrimps fed with commercial feeds and subjected to 20°C

CF35 - shrimps fed with commercial feeds and subjected to 35°C

SF20 - shrimps fed with commercial feeds + $1.0x10^8$ cell/g *L. salivarius* and subjected to 20°C

SF35 - shrimps fed with commercial feeds + $1.0x10^8$ cell/g *L. salivarius* and subjected to 35°C

Basal values for the metabolic rate (see 2.3) and antioxidant assays were determined after the acclimatization period. Each group was cultured and fed with their respective diets for nine (9) days. The analysis was performed every three days, starting from the feeding, collecting three shrimps per group. The CF20 and SF20 groups were transferred in a plastic container with 20°C dechlorinated water whereas groups CF35 and CF35 were placed in a plastic container with 35°C dechlorinated water. The shrimps were subjected to these temperatures for three hours while measuring the parameters for metabolic rate determination (see 2.3). After three hours, the hepato-pancreas and mus-

cle of each shrimp were collected and processed accordingly. Similar antioxidant assays were also performed in order to evaluate the antioxidant enzyme levels of the shrimps.

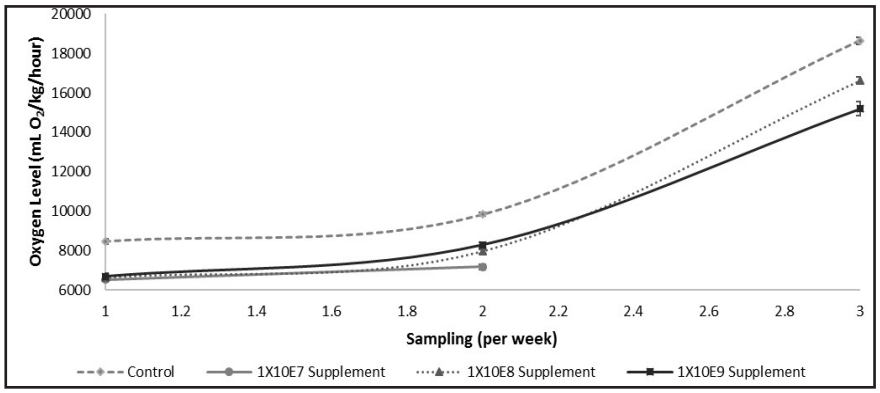

Figure 1. Oxygen level in the set-up of *M. rosenbergii* fed with commercial feeds and different cell density of *L. salivarius* (cells/g feeds). Results are reported as mean±SEM, where n=3. Data between metabolic rates and treatments were significantly different with $p < 0.05$.

2.6 Statistical Analysis

Statistical analysis and tools used in the experiment were computation for mean data and standard error of the mean. One-way analysis of variance was also used in the comparison of sample means.

3. RESULTS AND DISCUSSION

3.1 Water Quality Assessment

Low water quality facilitates the growth of pathogenic bacteria affecting the survival and growth of shrimps whereas higher values that exceed the normal range required by the shrimps may affect the shrimp's metabolism and may induce oxidative stress in (Suantika et al. 2013). Table 1 summarizes the actual water condition during the experiment in comparison with the optimum range needed for normal shrimp metabolism. The table showed that the temperature and dissolved oxygen are within the normal range while pH, ammonia, and phosphates vary at some points. This is due to the presence of unconsumed feeds, ammonia excretion of shrimps and acidification of feeds caused by the microorganisms present in the water.

Table 1. Water quality parameters during the experiment as compared with the optimum range

Parameters	Optimum Range	Culture Range
Temperature	28-32	28-29
DO (mg/L)	5.0-9.0	5.0-7.0
pH	7.0-8.3	5.9-7.6
Ammonia (NH^3) (mg/L)	<0.03	0.5-4.0
Phosphate (PO_4^{-3}) (mg/L)		0.5-1.0

3.2 Effect of Probiotic Dietary Supplementation on the Oxygen Consumption of *M. rosenbergii* at Different Sampling Time and Temperature

The study was divided into two parts that include the evaluation and effect of different bacterial density ($1.0x10^7$, $1.0x10^8$, and $1.0x10^9$ cells/g) supplemented in feeds on the oxygen consumption of shrimps and the levels of antioxidant biomarkers after stress induction. The resulting supplemented feeds were mixed with potato starch that served as a binder for the feeds and bacteria. The feeds with starch as binder exhibit good adhesive property, good pelletability, hardness, water stability, and has minimum dust level, ensuring the stable quality of pellet that will not easily disintegrate in an aquatic medium and will reach until ingestion of the organism (Orire et al. 2010). Organisms, like shrimps, have a slow feeding habit that requires to use specific binders to prevent physical disintegration of feeds in the water for a certain period. Binders for shrimp feeds should have a high molecular weight for it to sink up to the bottom. Thus, the use of dietary binding agents that can undergo starch gelatinization such as cassava starch, potato starch, and flour are required to be used (Tacon 1987).

The oxygen consumption of shrimps fed with a different cell density of *L. salivarius* was presented in Figure 1, where four groups of shrimps were used in the experiment. The control group was fed with standard commercial feeds while the other shrimp groups were fed with commercial feeds supplemented with *L. salivarius* at $1.0x10^7$ (LS7), $1.0x10^8$ (LS8), and $1.0x10^9$ (LS9) cell/g of feeds (Fig. 1). The shrimps were fed every day for three weeks, and sampling periods were done once a week. The water temperature was noted to be at the range of 20-25°C throughout the experiment.

The oxygen consumption showed a gener-

al trend for all shrimp groups in the experiment. The value of oxygen consumption was found to be directly proportional to the sampling period or prolonged exposure to dietary supplementation on which the chemical processes by which *M. rosenbergii* used up to make energy increased as to sampling periods. From low to high metabolic rate was observed in all shrimps sampled in the experiment. The LS7 group has high mortality compared to other groups of shrimps and only reached a two-week life span in the experiment. The LS8 group, which expressed a similar metabolic rate with LS7 during the first to second sampling followed an increment on the metabolic rate value on the third sampling.

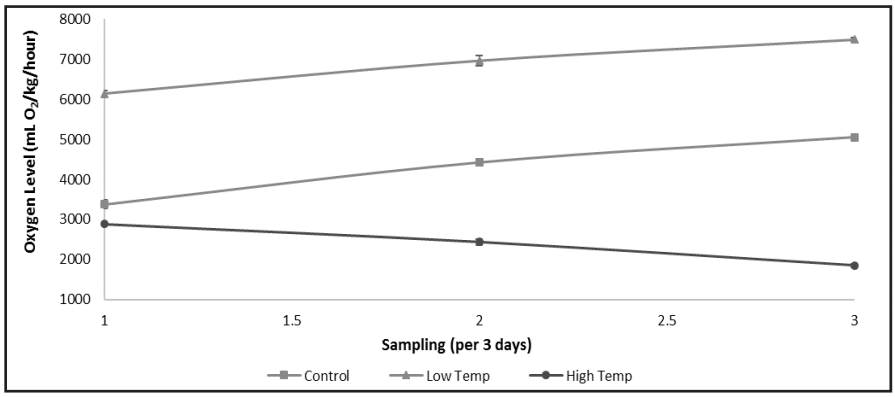

Figure 2. Oxygen level in the set-up of *M. rosenbergii* fed with commercial feeds and commercial feeds + $1.0x10^8$ *L. salivarius* cells/g feeds subjected to 20°C and 35°C. Results are reported as mean±SEM, where n=3 and $p < 0.05$.

Meanwhile, LS9 group showed a consistent increase of energy used up from the first up to the final sampling. The trend showed consistent pattern reaching the value close to the control group. Thereby, $1.0x10^8$ cell/g of feed supplementation was the most suitable and efficient group to use as a feed supplement for the shrimp in the temperature stress setup.

The effect of $1.0x10^8$, $1.0x10^9$ cells/g feeds, and the control on the oxygen consumption of the shrimp was directly proportional explaining that as the sampling time or prolonged feeding with dietary supplement increases the oxygen consumption of the shrimp also increases. It is notable that growth in terms of weight and length of shrimp can be associated on the weeks of sampling since it is continuously growing. Also, the metabolic rate could be associated with the growth of the shrimp since, during the development, shrimps will need and consume more oxygen, therefore metabolic rate increases. Notably, the result in Figure 1 supported that dietary supplementation of probiotics in the feeds of the shrimp promotes growth based on the oxygen consumption results.

Probiotic supplementations, which are bio-friendly agents from the lactic acid bacteria and *Bacillus sp.*, can be introduced into the culture environment of the shrimp or into the feeds to control the growth of pathogenic bacteria as well as promote growth on the shrimps by improving digestion of nutrients and helping on the survival rate of the cultured shrimps (Farzanfar 2006). Survival rate and growth of shrimps were also observed with the use of the following probiotic bacteria: *B. subtilis, L. sporogenes, S. cerevisae* (Seenivasan et al. 2012).

Based on the results obtained from the initial experiment, $1.0x10^8$ cell/g of feeds was used for the evaluation of the metabolic rate of shrimps subjected to low (20°C) and high (35°C) temperatures (Fig. 2).

Figure 2 showed the oxygen consumption of *M. rosenbergii* subjected to varying temperatures. The control group and SF20 groups presented a similar pattern in oxygen consumption. However, SF20 group had shown an increase in oxygen consumption compared with the SF35 group. It showed that metabolism increased linearly with low water temperature, but it decreased linearly with high temperature. The result was in contrast to the observed activity of Tidwell and Coyle (2002), where they demonstrated that there was a significantly lower shrimp survival at 25°C (24.3%) as compared with 20°C (96.48%) during transportation of *M. rosenbergii* and concluded that low water temperature could reduce shrimp metabolism, whereas as high temperature stimulates metabolism and increases the production of reactive oxygen species (ROS). Souza et al. (2013) noted that at low temperature, shrimp metabolism was reduced, and water quality was improved because of low excretion levels and oxygen consumption by shrimp.

According to Niu et al. (2003), effects of temperature on the food consumption growth, and even oxygen have been tested and noted that as the temperature of the environment increases, the oxygen consumption also increases. The views of Niu et al. (2003) were based on the Q10 principle in metabolic processes. Q10 is a measurement of temperature sensitivity of an enzymatic reaction or physiological pro-

cesses in a temperature change of 10°C. Q10 principle also explains that as the temperature increases the metabolic rate of reaction also increases, but this principle is somehow changed depending on the body temperature of the animal. Shrimp is an ectothermic animal on which it could not regulate its body temperature and rely on the temperature of the surroundings. Also, shrimps are classified under heterotherms on which it allows its body temperature to fluctuate. Shrimps have a characteristic of low metabolism and energy intake and have a wide temperature range for enzymatic activity. According to Dalla Via (1985), smaller shrimps tend to have higher rates of oxygen consumption than larger shrimps at 5 to 25°C and have lower Q10 values when exposed to increasing temperature.

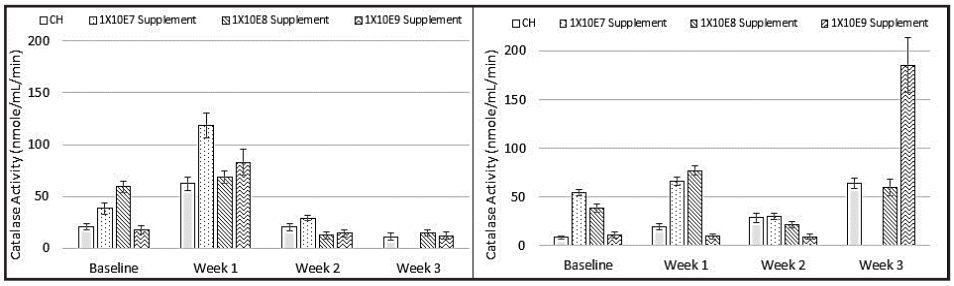

Figure 3. Catalase activity in the hepato-pancreas (A) and muscles (B) of shrimps fed with commercial feeds and different cell density of *L. salivarius* (cell/g of feeds). Results are reported as mean±SEM, where n=3 and data within each group of feed supplements are significantly different within supplements with $p < 0.05$.

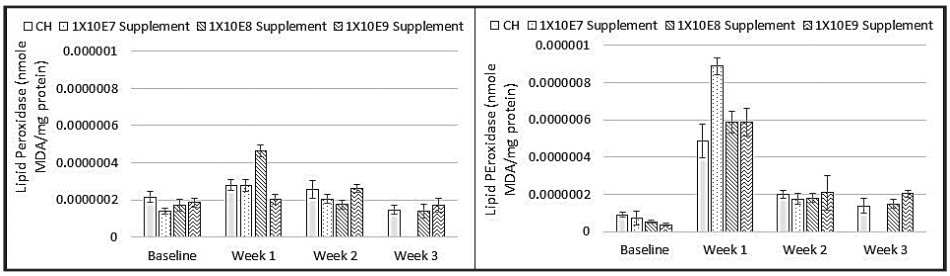

Figure 4. Lipid peroxidation as represented by malondialdehyde (MDA) concentration in the hepato-pancreas (A) and muscles (B) of shrimps fed with commercial feeds and different cell density of *L. salivarius* (cell/g of feeds). Results are reported as mean±SEM, where n=3 and data within each feed supplement are significantly different from each other with $p < 0.05$.

However, the observed pattern in this experiment may be attributed to the solubility of gases at varying temperature. Based on the gas law, the solubility of gases is inversely proportional to the temperature (Breck and Holmes, 1967). Thus, lower values of dissolved oxygen were recorded at the high temperature set up in contrast with the low temperature set up. Since dissolved oxygen values were used to compute for the metabolic rate (mL O_2/kg/hour), there will be a discrepancy in the result since dissolved oxygen may vary based on the temperature difference. Furthermore, the presence of *L. salivarius* might have affected the metabolic rate of the shrimps subjected to high temperature and somehow reduced the oxygen consumption of the shrimps.

3.3 Effect of Different Probiotic Dietary Supplementation on the Antioxidant Biomarkers

Reactive oxygen species (ROS) generation within a biological system have different kinds of mechanism, and they are usually formed as a by-product of oxygen metabolism (Luschak 2011). ROS can be produced from the reaction in the electron transport chain, oxygenases reaction, autooxidation, or other enzyme mechanistic pathways. However, these ROS produced are eliminated by scavenging activity and various REDOX reactions. These ROS can damage the cellular constituents and regulates cellular processes that can lead to adaptation or programmed cell death (Luschak 2011).

In this study, antioxidant biomarkers such as total glutathione (tGSH), lipid peroxides, and catalase activities (CAT) were measured in both the hepato-pancreas and muscles of the shrimps. Catalase enzyme is responsible for converting hydrogen peroxide (H_2O_2) to water (H_2O) and oxygen gas (O_2). This enzyme is considered to be a sensitive biomarker of oxidative stress (Mani et al. 2014), and the highest activity is recorded in the liver followed by the kidney, heart, brain, and muscle (Hidalgo et al. 2002).

As observed in Figure 3, CAT is higher in the hepato-pancreas during the first two weeks than in the muscles. However, there was an increase in CAT in the muscle during the 3rd week LS9 group. This observed pattern may be due to the multiple oxidative reactions and free radical generation occurring in the liver (Avci et al. 2005).

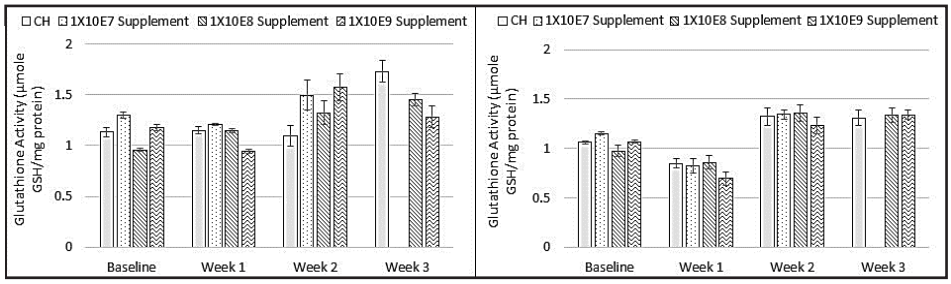

Figure 5. Total glutathione (tGSH) levels in the hepato-pancreas (A) and muscles (B) of shrimps fed with commercial feeds and different cell density of *L. salivarius* (cell/g of feeds). Results are reported as mean±SEM, where n=3. Data within each group were statistivcally different from each other with $p < 0.05$.

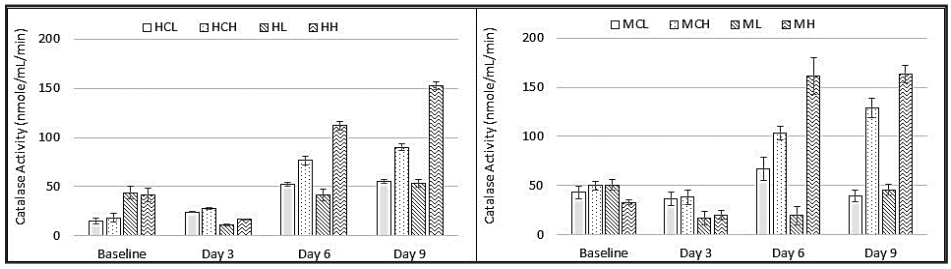

Figure 6. Catalase activity in the hepato-pancreas (A) and muscles (B) of shrimps fed with commercial feeds + $1.0x10^8$ *L. salivarius*/g of feeds. Results are reported as mean±SEM, where n=3. Data within each group are significantly different from each other with $p < 0.05$. (Note: HCL: Hepatopancreas-Commercial feed-Low temp (20°C); HCH: Hepatopancreas-Commercial feed-High temp (35°C); HL: Hepatopancreas-Supplemented feed-Low temp; HH: Hepatopancreas-Supplemented feed-High temp; MCL: Muscles-Commercial feed-Low temp; MCH: Muscle-Commercial feed-High temp; ML: Muscles-Supplemented feed-Low temp; and MH: Muscles-Supplemented feed-High temp)

LS 8 showed that upon supplementation on the shrimp, CAT activity on both hepatopancreas and muscles decreases as the week of supplementation increases. The lowering of CAT activity was due to the adaptation of the shrimp metabolism and innate immunity in the presence of probiotics in their system at a specific controlled cell number of bacteria.

Lipid peroxidation is also used as an indicator of oxidative stress in cells and tissues. The reaction involves the conversion of polyunsaturated fatty acid peroxides to malondialdehyde that forms an adduct with thiobarbituric acid (Garcia et al. 2005). Figure 4 shows that there was an increased lipid peroxidation during the first week of feeding with *L. salivarius* in all three concentrations both at the hepato-pancreas and in the muscle that eventually decreased during the 2nd and 3rd week of feeding. This may be due to the adaptation of the shrimp with the newly introduced feeds supplemented with bacteria.

Total glutathione (tGSH) level in hepato-pancreas and muscles showed that there was no difference between the tGSH levels of the two sample, but there is an evident increase in GSH levels during the 3-week feeding with *L. salivarius* (Fig. 5). The total glutathione content, together with glutathione-S-transferase and catalase, is one of the antioxidant defenses that is involved in counteracting the toxicity of ROS (Tu et al. 2012).

For the initial screening of the bacterial concentration, $1.0x10^8$ cell/g of feeds had the best effect towards antioxidant biomarkers as compared with $1.0x10^7$ cells/g and $1.0x10^9$ cells/g of feeds. As a whole, it has significantly increased CAT and lowered the lipid peroxidation but have a similar effect with the other experimental groups on the tGSH levels both in the hepato-pancreas and muscles of shrimps.

3.4 Effect of Probiotic Dietary Supplementation on Shrimp Exposed to Different Water Temperature as Stressor

To evaluate the effect of temperature in the antioxidant biomarkers, the researchers subjected the shrimps to 20°C and 35°C as a stressor for three hours. Results showed that there was a significant difference between the effect of low temperature and high temperature in the levels of antioxidant biomarkers.

Water temperature is one of the critical environmental factors that are being considered in aquaculture (Souza et al. 2013). Increase in temperature would stimulate various metabolic processes following the thermodynamic principles; thus, enhances oxygen consumption and increase ROS production resulting in oxidative stress (Lushchak 2011). On the other hand, oxidative metabolism might see a decrease in the induction of oxidative stress at low temperature, but in some cases, the low environmental temperature might cause oxidative stress especially when it comes to aquatic animals (Lushchak 2011). It was previously shown that at low temperature, there is

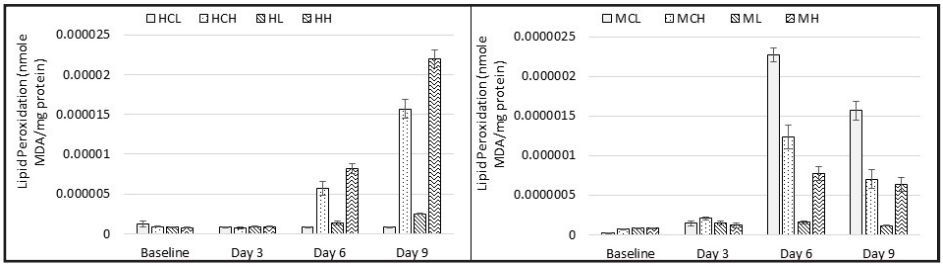

Figure 7. Lipid peroxidation as represented by malondialdehyde concentration in the hepato-pancreas (A) and muscles (B) of shrimps fed with commercial feeds + 1.0x10^8 *L. salivarius*/g of feeds. Results are reported as mean±SEM, where n=3. Data within each group are statistically different from each other with $p < 0.05$. (Note: CHL: Hepatopancreas-Commercial feed-Low temp (20°C); CHH: Hepatopancreas-Commercial feed-High temp (35°C); HL: Hepatopancreas-Supplemented feed-Low temp; HH: Hepatopancreas-Supplemented feed-High temp; CML: Muscles-Commercial feed-Low temp; CMH: Muscle-Commercial feed-High temp; ML: Muscles-Supplemented feed-Low temp; and MH: Muscles-Supplemented feed-High temp)

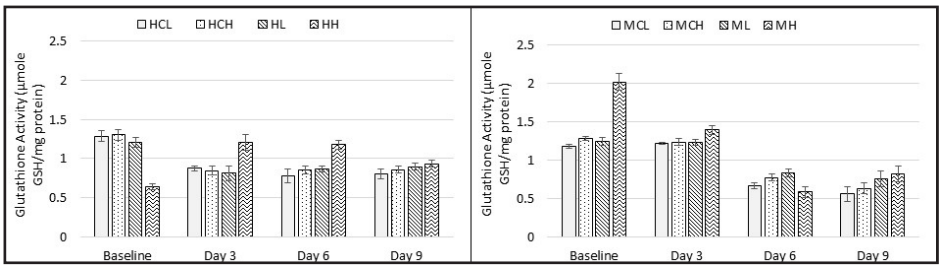

Figure 8. Total glutathione (tGSH) levels in the hepato-pancreas (A) and muscles (B) of shrimps fed with commercial feeds + 1.0x10^8 *L. salivarius*/g of feeds. Results are reported as mean±SEM, where n=3. Data within each group are statistically different from each other with $p < 0.05$. (Note: HCL: Hepatopancreas-Commercial feed-Low temp (20°C); HCH: Hepatopancreas-Commercial feed-High temp (35°C); HL: Hepatopancreas-Supplemented feed-Low temp; HH: Hepatopancreas-Supplemented feed-High temp; MCL: Muscles-Commercial feed-Low temp; MCH: Muscle-Commercial feed-High temp; ML: Muscles-Supplemented feed-Low temp; and MH: Muscles-Supplemented feed-High temp)

a lower production of ROS and thus, lower oxidative stress is being experienced by shrimps (Tidwell and Coyle 2002). The CAT levels (Fig. 6) in both the hepato-pancreas and muscle of shrimps subjected to 20°C were not significantly different.

This would imply that the shrimps did not experience too much oxidative stress due to their lower metabolism at a lower temperature. In contrast, there was an observed increase in CAT levels both at the hepato-pancreas and muscle of shrimps subjected to 35°C, indicating that the shrimps undergo oxidative stress due to the sudden increase in temperature. The results are in accordance with the study conducted by Souza et al. (2013) where they demonstrated that at higher temperature there was an increase in CAT levels due to the increase of metabolism in shrimps.

On the study of Chen et al. (2017), the prawn (*Macrobrachium rosenbergii*) was fed with prebiotic, Fructooligosaccharide (FOS), and showed antioxidant biomarkers activities like lipid peroxidation and superoxide dismutase (SOD). The prawns fed with 1% to 2% dietary FOS showed oxidative stress and reduced the hepatopancreatic condition. Increasing dietary FOS increased the lipid peroxidation activity significantly and decreased the SOD activity.

However, this was in contradictory to the results of Vinagre et al. (2014) that showed a decrease in CAT level at temperatures ranging from 28-32°C. The researchers then explained that at this specific temperature range, the catalase enzyme might be consumed at a faster rate rather than being produced.

Another antioxidant biomarker that was significantly affected by the temperature change is the lipid peroxidation. As observed in Figure 7, there was an increasing trend of lipid peroxidation in the hepato-pancreas of shrimps that were subjected to high temperature. Since the experiment was conducted only for two weeks, there was a possibility that during this period the shrimps are still adapting to their new diet, making the lipid peroxidation more pronounced due to the double effect of temperature and bacterial stress. On the other hand, lipid peroxidation was significantly decreased in the muscle of shrimps subjected to low temperature and fed with *L. salivarius* supplement as compared with the control.

However, the tGSH levels in the hepato-pan-

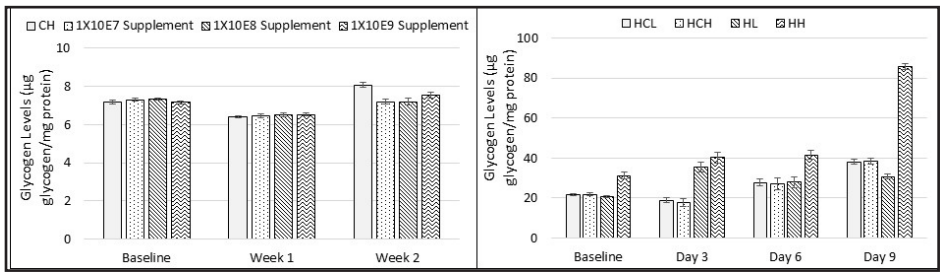

Figure 9. Glycogen levels in the hepato-pancreas of shrimps fed with (A) commercial feeds with a different cell density of *L. salivarius* (cell/g of feeds) and (B) commercial feeds + 1.0x10^8 *L. salivarius*/g of feeds. Results are reported as mean±SEM, where n=3. The data within each group are statistically different from each other with $p < 0.05$. (Note: HCL: Hepatopancreas-Commercial feed-Low temp (20°C); HCH: Hepatopancreas-Commercial feed-High temp (35°C); HL: Hepatopancreas-Supplemented feed-Low temp; HH: Hepatopancreas-Supplemented feed-High temp)

creas and muscles of the shrimp have no significant and evident increase in GSH levels during the duration of feeding with 1.0x10^8 *L. salivarius* cells per gram of feeds (Fig. 8).

In this study, glycogen levels in shrimps that were fed with commercial feeds supplemented with different concentration of *L. salivarius* were measured. As shown in Figure 9, there was no significant difference between the glycogen concentrations in the hepato-pancreas of each group thereby, the presence of *L. salivarius* does not affect the carbohydrate metabolism of the shrimp at any bacterial concentration.

On the other hand, there was a significant increase in the glycogen levels of the HH groups as compared to the control group subjected to high temperature (HCH) during the 9th day. This result would indicate that there was an increase in carbohydrate metabolism during the period where the shrimps were subjected to high temperature.

Glycogen plays a vital role as a source of glucose supply found in the hepato-pancreas (Rosas et al. 2000) and is also used in the formation of the exoskeleton during the molt cycle of the shrimp. Recently, shrimp physiology has been related to diet manipulation (Rosas et al. 2000) to provide a better understanding of their carbohydrate utilization and to detect physiological stress.

4. CONCLUSION

The results indicate that 1.0x10^8 cell/g of supplemented dietary feeds can improve the metabolic rate and lessen the oxidative stress level based on the activity in lipid peroxidation, glutathione, and catalase. Therefore, regular feeding of feed supplemented with *L. salivarius* may help in the development and metabolism of *M. rosenbergii* as well as lessening its susceptibility towards infection by boosting the oxidative response of the shrimp. However, further studies are still needed to establish and confirm the effective dose for a more efficient effect on a commercial scale application. Feed supplementation with probiotic microorganisms will help the development of metabolism and immune system of the shrimp in response to environmental stress and infection. This will be very beneficial to the shrimp industry of the Philippines since the country is experiencing a continuous change in the environment, which is the effect of global warming and advance strain of infectious microorganisms. Since the Philippines is a tropical country, which is a haven of sudden temperature change and diverse microflora, probiotic treatment and supplementation of feeds in the aquaculture industry will have a significant impact on the development, metabolism, and sustained growth.

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