ADDITIVE EFFECTS OF POLY- β -HYDROXYBUTYRATE ON GROWTH AND IMMUNE RESPONSES OF JUVENILE NILE TILAPIA *OREOCHROMIS NILOTICUS*, HYBRID STRIPED BASS *MORONE CHRYSOPS* × *M. SAXATILIS*, AND RED DRUM *SCIAENOPS OCELLATUS* BASED ON *IN VIVO* AND *IN VITRO* APPROACHES

A Thesis

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BLAINE A. SUEHS

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Chair of Committee,	Delbert M. Gatlin, III
Committee Members,	Guoyao Wu
	Thomas H. Welsh, Jr.
Interim Head of Department,	Kirk Winemiller

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ABSTRACT

Disease outbreaks in intensive aquaculture, particularly from bacterial pathogens, represent major constraints to efficient fish production, necessitating development of novel and innovative disease treatment and prevention strategies. Poly-β-hydroxybutyrate (PHB), a biopolymer synthesized by specific gram-negative and gram-positive bacteria, is a compound with potential immunostimulatory capabilities for various fish species. This study analyzed the efficacy of PHB as an immunomodulator in Nile tilapia, hybrid striped bass (HSB), and red drum based on both *in vitro* and *in vivo* approaches. *In vitro* immunological assays, namely intra- and extra-cellular superoxide anion production of head-kidney-derived macrophages exhibited significant ($P<0.05$) linear and quadratic relationships when graded doses of 3-hydroxybutryate (3HB) (0.0, 0.5, 1.0, 2.0, 4.0 and 8.0 mM) were added to Nile tilapia cells in culture media. In contrast, intra- and extra-cellular superoxide anion production and bactericidal capacity of HSB head-kidney-derived leukocytes were determined to be significantly ($P < 0.05$) reduced when identical graded doses of 3HB was supplemented in the cell culture media. For the *in vivo* feeding trials, PHB-synthesizing bacteria, *Zobellella denitrificans*, were produced on-site at the Texas A&M Aquacultural Research and Teaching Facility and then supplemented to species-specific basal diets to produce five isonitrogenous and isolipidic experimental diets containing PHB in stepwise increments (0.125, 0.25, 0.5, 1.0, and 2.0% of dry-diet weight). In addition, a control diet was supplemented with 0.5% of a commercial purified PHB product. Juvenile Nile tilapia, HSB, and red drum (~ 1.3, 5.5, and 4.4 g/fish initial weight, respectively) in separate feeding trials were stocked in 38-L aquaria operated as a recirculating aquaculture system, complete with settling chamber, biological and sand filtration, and UV sterilization. Each of the experimental diets was randomly assigned to quadruplicate aquaria of fish and fed for an 8-week period. Nile tilapia exhibited significant

($P<0.05$) dose-dependent linear and quadratic relationships with regard to percentage weight gain, as well as feed efficiency, protein conversion efficiency, and hepatosomatic index, while HSB and red drum exhibited limited responses in these growth parameters. Alternatively, only juvenile red drum showed significantly ($P<0.05$) increased muscle yield ratios with increasing dietary PHB while whole-body proximate composition analyses revealed no significant ($P>0.05$) differences in any feeding trial. Thus, a species-specific effect of PHB was observed for growth parameters, condition factors, and immunological responses of Nile tilapia, HSB, and red drum juveniles, with Nile tilapia exhibiting the most dramatic, positive responses.

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1. INTRODUCTION

Aquaculture represents the fastest growing food-production sector in the world, producing a record 114.5 million tonnes (MMT) of live product worldwide in 2018 (FAO, 2021). Put in perspective, the beef industry reportedly produced approximately 61 MMT of bovine meat products in 2018 (FAO, 2020). To continue to exceed milestone numbers observed in long-established sectors of the livestock industry, aquaculture must overcome unique pitfalls and challenges specifically related to the high dietary protein requirements of most fish species. Fully exploited fisheries have caused a plateau in capture fisheries, forcing aquaculture to search for and adopt suitable alternative protein ingredients to adequately replace traditional fishmeals and other marine ingredients (Alder et al., 2008; Naylor et al., 2009). In addition, disease prevalence, particularly bacterial pathogens associated with intensively reared aquatic species must be addressed to further expand industry outputs (Iguchi et al., 2003; Shoemaker, Evans, & Klesius, 2000). Novel and innovative disease treatment and prevention strategies without dependence on antibiotics are essential for aquaculture and other food-production industries to sustain the increasing human population which is estimated to reach 9 billion by the year 2050 (Béné et al., 2015).

1.1 Streptococcus iniae and Aeromonas hydrophila Impacts on Aquaculture

Aquatic disease outbreaks represent a major barrier to more efficient fish production for most all sectors of aquaculture. For example, annual economic losses due to bacterial infections in China, the world's leading producer of aquatic species, amounted to 15% of total stock's worth (FAO, 2021; Qi, 2002). *Streptococcus iniae* and *Aeromonas hydrophila* are major bacterial pathogens presently contributing to aquatic disease outbreaks. *Streptococcus iniae*, initially

isolated from rainbow trout in 1958 (Hoshina, 1958), is a gram-positive bacterium with documented infections in 27 species of marine and freshwater finfish species (Agnew & Barnes, 2007). *Aeromonas hydrophila*, on the other hand, is a gram-negative motile bacterium capable of causing enteric septicemia, hemorrhagic symptoms, and chronic ulceration (Cipriano, Bullock, & Pyle, 1984; Nicholson et al., 2020). The typical practice of high-density aquaculture allows rapid onset and prevalence of these pathogens, which often lead to increased mortality. As Shoemaker et al. (2000) observed, Nile tilapia stocked at 22.4 g/L and infected with *Streptococcus iniae* succumbed to the pathogen at much higher rates compared to other experimental groups at lower densities. Also worth noting was the stocking densities used by Shoemaker et al. (2000) were well below stocking densities commonly used at commercial facilities, suggesting an even greater effect of production settings on disease incidence. Zoonotic outbreaks also have been recorded for each of these bacterial species (González-Serrano et al., 2002; Lau et al., 2006), presenting a pressing need for effective prevention strategies to limit human illness and reduce economic losses.

Although effective in theory, antibiotics are decreasingly used in practice due to substantial expense to farmers, co-evolved immunity of the pathogen, environmental impacts, and potential bioaccumulation in consumers. Then again, vaccines present a separate challenge due to limitations in application procedures and variability in bacterial serotypes which may render vaccinations ineffective (Agnew & Barnes, 2007). The evolution of novel viral pathogens also has presented a distinctive challenge in identifying preventative measures, as seen by the global epidemic outbreak of the tilapia lake virus (TiLV) (Nicholson et al., 2020).

One area of research in disease prevention has focused primarily on dietary additives capable of stimulating the innate immune response of fish and proactively suppressing costly outbreaks.

The Fish Nutrition Laboratory at Texas A&M University has pursued this area for several years. To date, effective immunomodulation has been observed with dietary additives such as nucleotides (de Cruz et al., 2020), certain amino acids such as arginine and glutamine (Chen et al., 2015; M. Li et al., 2016), β-glucans (Yamamoto et al., 2020; Yamamoto et al., 2018), oligosaccharides (Hoseinifar et al., 2015), and organic acids (Ebrahimi et al., 2017; Mendoza Rodriguez, Pohlenz, & Gatlin III, 2017). However, many of these additives are costly and may not be effective in an intensive aquaculture setting. Therefore, a cost-effective ingredient to combat disease incidence in aquaculture is needed with limited environmental and secondary biological impacts.

1.2 Poly-β-hydroxybutyrate Function and Application

Research in aquaculture has recently focused on poly-β-hydroxybutyrate (PHB), the most common of the polyhydroxyalkanoate (PHA) molecules. Such research has demonstrated its ability to serve as an immunostimulant for various fish and crustacean species (Franke et al., 2017; Kiran et al., 2020; Laranja & Bossier, 2020). PHB is a microbial synthesized polymer that serves a variety of physiological and structural purposes, including carbon and energy storage for bacteria under nutrient limitations and as a promising biodegradable thermoplastic for human uses (Dawes, 1988; Hankermeyer & Tjeerdema, 1999). PHB is produced by gram-negative and gram-positive bacteria such as *Zobellella denitrificans* and *Alcaligenes eutrophus*, offering variable options for upscaling production of this polymer as a feed additive for commercial application (Asiri & Chu, 2020; Kavitha, Rengasamy, & Inbakandan, 2018).

Mechanisms of PHB immune stimulation are based on the compound's ability to be degraded into short-chain fatty acids (SCFAs), such as butyrate, by the secretion of PHB depolymerase enzymes from bacteria in the gastrointestinal tract of aquatic organisms (Liu et al., 2010). Coupled

with acting as an additional energy substrate for leukocytes and other immune cells and increasing enterocyte proliferation and regeneration in the intestine (Liu et al., 2014), these fatty acids ultimately may penetrate bacterial cell membranes and lower cytoplasmic pH to more acidic conditions, thus halting the growth and proliferation of pathogenic bacteria (Koh et al., 2016; Laranja & Bossier, 2020; Ng & Koh, 2017). Along with immunomodulation, SCFAs have been observed to increase mineral availability in aquatic diets by supporting pH reduction in the gastrointestinal tract, and chelating calcium cations in the gut (Hoseinifar, Sun, & Caipang, 2017; Ravindran & Kornegay, 1993). Specifically, these findings are supported by Koh et al. (2016) in which hybrid red tilapia exhibited significantly higher growth performance, bactericidal capabilities, and digestibility of phosphorus and dry matter when fed diets containing 0.5 and 1.0% of an organic acid blend consisting of formic acid, lactic acid, malic acid, tartaric acid and citric acid compared to a practical control diet. More recent advances concerning PHB supplementation indicate potential benefits during periods of high stress, specifically sexual reversion in tilapia (Jesus et al., 2019). To my knowledge, the rate of PHB oxidation as an energy substrate in fish tissue has not been measured in the same capacity as amino acids such as glutamate, glutamine, and aspartate (Li et al., 2020). However, as previously stated, prior comparative feeding trials reported increased growth as PHB inclusion increased, suggesting an increase of PHB oxidation in tissues. Thus, PHB has the potential to enhance not only immune response of cultured species, but growth performance and nutrient digestibility as well.

Nonetheless, PHB is a biopolymer with many potential applications at various stages of intensive aquaculture. For example, Asiri and Chu (2020) proposed a novel recirculating aquaculture system using particulate waste in the settling chamber to effectively proliferate PHB-producing *Zobellella denitrificans* bacteria. The produced PHB could then be used as a feed

additive with the process repeated for sustainability. In addition, PHB has been proposed as a suitable carbon source for denitrifying bacteria in recirculating systems and next-generation biofloc production (Gutierrez-Wing, Malone, & Rusch, 2012; Luo et al., 2017; Zhang et al., 2014). It is evident that PHB is a molecule capable of numerous functions and applications, from water quality enhancement to feed supplementation, and should be studied further.

1.3 Species-Specific Aquaculture Production

Nile tilapia *(Oreochromis niloticus)* ranks as the third most cultured fish in the world at 8.3% of total production in 2018 (FAO, 2021). This species is native to northern Africa but has become economically viable for world aquaculture production in various regions of the world such as Asia, Central America and South America, as well as countries including Ghana, China, and the United States to name a few (Asiedu, Failler, & Beyens, 2016; El-Sayed, 2019). Due to its high tolerance to most rearing conditions in various types of culture systems, many speculate that tilapia will be a model organism for developing next-generation aquaculture practices (Yue, Lin, & Li, 2016). As such, research evaluating compounds geared to enhance immune function and disease resistance of Nile tilapia, especially in high-density systems, is warranted.

Hybrid striped bass (*Morone chrysops x M. saxatilis;* HSB) is a hybridized fish produced from the white bass and striped bass that thrives in a wider range of environmental and water quality conditions than the parental species, namely temperatures from 4 to 36°C and salinities of up to 25 parts per thousand (Hodson, 1990). This carnivorous species is of particular importance to United States aquaculture as both a popular recreational sport fish and food fish (FAO, 2021; Hodson, 1990). General advantages of this genetic cross over the parent lineages include increased tolerances to less optimal environmental conditions, increased disease tolerance, improved growth,

and overall improvement in acceptance of pelletized diets (Garber and Sullivan, 2006). As HSB production has increased over the past 4 decades (FAO, 2021; Gempesaw II et al., 1992), it is imperative to proactively mitigate disease outbreaks to this fish. Thus, research into dietary additives, specifically PHB supplementation is warranted to support the continued expansion of HSB production.

Similarly, red drum (*Sciaenops ocellatus*) is a popular euryhaline species of commercial value through culture as a food fish and also as a highly sought-after sport fish (Robinson, 2017). Although global production of Sciaenids (~71,000 tons in 2016) (FAO FishStat, 2016) is primarily located in Asia, and not as prevalent as several other marine finfish, the production of red drum is vital to stock enhancement and food fish production in the state of Texas (Gatlin, 2002). Texas Parks and Wildlife reports total production of larval and juvenile red drum released for state-sponsored stock enhancement programs to be at eight hundred million fish since inception of the hatchery program in 1983 (TPWD.gov, 2021). Because red drum are cultured intensively for both stock enhancement and food, the potential of enhancing growth and disease resistance with dietary PHB deserves consideration.

1.6 Specific Objectives

It is evident that PHB is a compound with many applications, especially regarding immuno-enhancement. Because of the potential for multiple uses of PHB and the popularity of commercially farmed Nile tilapia, hybrid striped bass, and red drum, research was pursued to further characterize the mechanistic actions of this compound. To accomplish such, the objectives of this research were to: 1) Evaluate *in vitro* immunological responses of head-kidney-derived leukocytes and peripheral-blood lymphocytes from each of those fish cultured in the presence of

graded doses of PHB 2) Evaluate the growth performance, feed efficiency, and condition indices of juvenile Nile tilapia, hybrid striped bass, and red drum fed diets with incremental levels of PHB by comparative feeding trial; ; and 3) Establish the optimal inclusion level of PHB in the diets of juvenile Nile tilapia, hybrid striped bass and red drum.

2. MATERIALS AND METHODS

2.1 In vitro Assays

To confirm pathogenic bacterial inhibition capacity of the PHB molecule, minimum inhibitory concentrations of (±) sodium 3-hydroxybutyrate (3HB, cat#54965, Sigma-Aldrich), a derivative of PHB, were assessed using *Aeromonas hydrophila* in acidic and neutral culture media. Briefly, brain heart infusion (BHI, cat#53286, Sigma-Aldrich) agar plates of specific pH (6.0 and 7.0) were plated with BHI broth cultured *Aeromonas hydrophila* and then infused with disks of graded concentrations of 3HB (0.0, 12.5, 25.0, 50.0, 100.0, and 200.0 mM). pH of the respective cell culture medias was confirmed by a precision pH probe (Mettler Toledo, Columbus, OH, USA). Areas of inhibition were monitored, and diameters measured at 2-h intervals for a duration of 14 h.

2.1.1 Head-Kidney Macrophage Isolation

In vitro assays with Nile tilapia and HSB head-kidney-derived macrophages were conducted using similar procedures. The head-kidney-derived macrophages were obtained from advanced stage Nile tilapia and HSB routinely maintained at the Aquacultural Research and Teaching Facility (ARTF) but separate from fish used in the *in vivo* feeding trials. These tilapia and HSB were fed species-specific commercial diets consisting of either 32 and 42% crude protein, respectively, and kept indoors in 1200-L fiberglass round tanks fashioned as a

recirculating aquaculture system, complete with settling chamber, biological filtration, sand filter, and UV sterilizer. Water quality was kept within desirable ranges to prevent macrophage and lymphocyte degradation prior to collection.

Fish were then anaesthetized in a solution of MS-222 at 100 mg L^{-1} prior to collection of blood through the caudal peduncle vasculature using heparinized syringes. After blood collection, fish were then euthanized in 300 mg L^{-1} MS-222 and ventrally dissected to remove head kidneys. Collected head kidneys were subsequently placed in Leibowitz cell culture media (L-15, cat #L5520, Sigma Aldrich) containing 5% fetal calf serum for leukocyte isolation. Lymphocytes were isolated from whole blood diluted 1:2 in Hank's Balanced Salts Solution (HBSS) and centrifuged at 350 x *g* using a layered gradient of 51 and 34% Percoll solution (cat #P1644, Sigma-Aldrich) and washed using a 1X solution of HBSS for the proliferation assay (Carvalho et al., 2018; Miller & Clem, 1988). Macrophages were isolated from head kidneys using procedures similar to lymphocyte isolation as described by Miller and Clem (1988) with modifications of Carvalho et al. (2018). Head kidneys were mechanically homogenized using a Dounce homogenizer, washed with HBSS, and isolated by Percoll gradients centrifuged at 350 x *g*.

The following assays were subsequently conducted following macrophage and lymphocyte isolation to assess innate immune response capabilities of juvenile Nile tilapia and HSB immune cells when incubated with graded concentrations (0, 0.5, 1.0, 2.0, 4.0, and 8.0 mM) of 3HB in the culture media. Initially, a higher 3HB concentration was utilized as determined by the optimal bacterial inhibition level observed at 50 mM, but was reduced to the concentrations listed above when immediate immune cell inhibition was observed.

2.1.2 Respiratory Burst of Head-Kidney-Derived Leukocytes

Respiratory burst of isolated head-kidney-derived leukocytes (a common type of macrophage) was measured via intracellular and extracellular superoxide anion production/concentration as described by Secombes (1990) and modified by Carvalho et al. (2018). Briefly, purified leukocytes were pipetted in two separate 96-well microplates to reach a concentration of 1.0 x 10^7 and incubated overnight in the various concentrations of 3HB culture media. A total of 12 replicate wells per 3HB concentration were prepared.

To measure intracellular superoxide production, 100 µL of solution containing 1 mg/mL of nitroblue tetrazolium (NBT) (cat# 97061–412, VWR International) and 1 µg/mL of phorbol 12-myristate 13-acetate (PMA, cat #P8139, Sigma) was added to all wells of a 96-well microplate containing the desired concentration of cells previously mentioned. After a 45-minute incubation, cells were washed twice with pure methanol (MeOH) and fixed. Formazan crystals were then dissolved by addition of 120 µL of 2 M KOH and 140 µL of dimethyl sulfoxide (DMSO, cat #D8418, Sigma Aldrich) to produce a turquoise hue that was read at 620 nm on a spectrophotometer.

Extracellular superoxide concentration analysis was performed by the addition of 100 µL of solution containing 2 mg/mL of cytochrome *c* and 1 µg/mL PMA to seven rows of the 96-well microplate containing 1.0 x 10^7 leukocytes. The final row constituted the negative control by adding 100 µL of the previous solution, but with 300 u/mL superoxide dismutase (SOD) added to halt superoxide anion formation. The microplate was immediately read at 550 nm to assess baseline absorbance and then read every 10 minutes until absorbance in the wells without the inclusion of SOD ceases to increase.

2.1.3 Peripheral-Blood Lymphocyte Proliferation

Peripheral blood lymphocytes from both Nile tilapia and HSB also were isolated and incubated overnight in culture media containing specified doses of 3HB according to procedures described by Miller and Clem (1988). Isolated lymphocyte proliferation was measured based on techniques described by Mosmann (1983) and modified by Carvalho et al. (2018). Ultimately, lipopolysaccharide solution (LPS, cat #L2630, Sigma Aldrich, 10 mg mL^{-1}) was used to stimulate lymphocyte proliferation and measured by the addition of 3-(4,5-dimethyl-2thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT, cat#M6494, Thermo Fisher Scientific) and read at 570 nm after extended incubation. The stimulation index was computed as a ratio of the absorbance of stimulated cells over the absorbance of non-stimulated (control) cells.

2.1.4 Bactericidal Assay

The ability of macrophages to kill *Streptococcus iniae* was measured based on methods developed by Secombes (1990). Briefly, after leukocytes were accurately concentrated at the desired level and incubated overnight with specific concentrations of PHB, *Streptococcus iniae* bacteria (at approximately $1x10^8$ cells or 0.5 optical density at 540 nm) were added at 20 µL to each well of a 96-well microplate and incubated at times of 0 (control) and 2.5 h to assess real-time killing ability. After each incubation, supernatants were removed and the macrophages selectively lysed with 0.2% Tween 20 (cat #H285, Mallinckrodt) leaving bacterial cells intact. After lysing macrophages, surviving bacterial cells were re-suspended in BHI broth and incubated further for 18 h. After incubation, MTT was added and plates read at 620 nm. Bactericidal capabilities of the macrophages were calculated based on a standard curve of bacteria concentration by percentage of absorbance of the control previously read at

0 h and the reading after incubation; % killing = bacteria 0 h - bacteria 2.5 h×100 ÷ bacteria 0 h (Carvalho et al., 2018). Macrophages with greater bactericidal capabilities allowed fewer surviving bacteria after lysing.

2.1.5 Blood Neutrophil Oxidative Radical Production

During termination of the feeding trials (described below), whole-blood samples were aliquoted in 5-mL tubes and incubated with 2 mg of NBT mL^{-1} in a 96-well microplate for 30 minutes prior to addition of dimethyl sulfoxide (DMSO, cat #D8418, Sigma Aldrich) and further incubation (Siwicki, Anderson, & Rumsey, 1994). Samples incubated in the DMSO solution were then centrifuged at 5,000 x *g* for 5-minutes followed by plating of 200 µL $sample^{-1}$ in a 96-well microplate. Blood neutrophil oxidative radical production was then measured at 545 nm using a microplate spectrophotometer.

2.2 Zobellela denitrificans (ZD1) Production

In preparation for the comparative feeding trials described below, an inoculant of *Zobellella denitrificans (*ZD1*)* bacteria was provided by the Chu Biotechnology Laboratory of the Texas A&M University's Department of Civil Engineering. Bacteria used in the feeding trials were inoculated and produced on site at the Texas A&M University System ARTF with the assistance of Dr. Fahad Asiri of the afore mentioned Chu Laboratory. Several 40-L Nalgene containers (Nalgene Inc., Rochester, NY) were filled with 20 L of deionized water and a solution of macro- and micronutrients previously developed by Asiri and Chu with a salinity of approximately 22 ppt (Asiri and Chu, 2020). Oxygenation was provided by aeration stones attached to an electrical blower to satisfy the requirement of this heterotrophic bacteria as well as maintain proliferating cells in suspension to allow for optimal growth (Asiri and

Chu, 2020). The ZD1 bacterial broth culture was inoculated in the containers at 4.0% of total volume and allowed to proliferate for 3 days. On the third day, bacteria were harvested by chemical flocculation with chitosan (50 mg/L) at a pH of 7.2, and the collected bacterial cells were freeze dried and ground with mortar and pestle for diet incorporation and evaluation in three separate feeding trials. The poly-hydroxy butyrate concentration of ZD1 bacteria was measured using gas chromatography by Fahad Asiri and verified to be approximately 21.0 g/100 g of dry weight. ZD1 incorporation in the experimental diets was adjusted to ensure PHB concentration was at the desired level based on the concentration of PHB in lyophilized cells.

2.3 Diet Formulation and Experimental Design – Nile tilapia feeding trial

Seven isonitrogenous and isolipidic diets for juvenile Nile tilapia (feeding trial 1) were formulated to contain 36% crude protein and 6% lipid, based on practical ingredients but low in fishmeal similar to that used in previous trials with juvenile Nile tilapia in this laboratory (e.g., Suehs and Gatlin, 2022) and well established in the literature (Koch et al., 2016). The basal diet did not contain PHB-producing ZD1 bacteria, while five experimental diets were supplemented with lyophilized ZD1 at 0.55, 1.10, 2.15, 4.30, and 8.60 g/100 g of dry diet weight in place of cellulose (Table 1), resulting in PHB concentrations of 0.125, 0.25, 0.5, 1.0, and 2.0% of dry weight, respectively. In addition, another basal diet was supplemented with a commercial PHB ingredient (Goodfellow Corporation, Coraopolis, PA, USA) at 0.5% of dry weight. The experimental diets met all nutrient requirements of juvenile Nile tilapia in accordance with NRC (2011) recommendations.

Experimental diets for this and the other two trials described below were produced on site at the ARTF by homogenizing dry ingredients in an industrial V-mixer prior to oil and water

addition and then pelleting into 3-mm strands using a commercial Hobart mixer (Hobart Corporation, Troy, OH, USA) as previously described by Yamamoto et al. (2018). Samples of each diet were subjected to PHB quantification analysis by gas chromatography according to methods described by Law and Slepecky (1961) with modifications by Asiri and Chu (2020). Proximate composition of the dried diets was measured in accordance with AOAC (2005) methods. Briefly, crude protein was measured by a LECO (St. Joseph, MI, USA) 828 Nitrogen and Protein Determinator, crude lipid content was determined by chloroform-methanol extraction (Folch et al., 1957), and ash/organic matter content was determined by combustion in a furnace at 650 C for 3.5 h.

For feeding trial 1, juvenile Nile tilapia produced from stocks at the ARTF were acclimated and hardened for a period of 1 week in 30, 38-L aquaria set up as a recirculating aquaculture system, comprised of settling chamber, biological filtration, sand filter, and UV sterilizer. During this 1-week period, fish were fed a commercial diet (Rangen, Angelton, TX) containing 32% crude protein. After the conditioning period, fish were then accurately weighed as groups of 15 fish per aquarium, and evenly distributed in each of the 30 aquaria to have similar initial weights (1.3 g initial weight ± 0.05 SEM). Each diet was randomly assigned to sextuplicate groups of juvenile Nile tilapia and fed twice daily at a set percentage of body weight to approach apparent satiation without overfeeding throughout the 8-week feeding trial. Weight gain was monitored weekly by group weighing all fish in each aquarium and adjusting rations equally among diets based on observed feeding behavior to minimize accumulation of uneaten feed in the recirculating aquaculture system. Water quality was analyzed twice a week, and parameters were kept within ranges suitable for Nile tilapia culture including the following (mean + SEM): Temperature (˚C):

29.2 ± 0.4; O_2 (mg L^{-1}): 7.31 ± 0.5; TNN (mg L^{-1}): 0.009 ± 0.01; TAN (mg L^{-1}): 0.09 ± 0.05; Salinity (ppt): 1.49 ± 0.5; pH: 8.22 ± 0.2 (average ± standard deviation).

Table 1: Formulations of the experimental diets containing graded levels (g/100g) of PHB from lyophilized *ZD1* or a purified PHB ingredient fed to Nile tilapia (feeding trial 1) juveniles for 8 weeks.

Ingredients	Basal	Commercial PHB Ref.	ZD1 PHB 0.125	ZD1 PHB 0.25	ZD1 PHB 0.5	ZD1 PHB 1.0	ZD1 PHB 2.0
Soy protein concentrate [1]	13.25	13.25	13.25	13.25	13.25	13.25	13.25
Menhaden fishmeal [2]	7.45	7.45	7.45	7.45	7.45	7.45	7.45
Dehulled soybean meal [3]	37.85	37.85	37.85	37.85	37.85	37.85	37.85
Wheat flour [4]	13.85	13.85	13.85	13.85	13.85	13.85	13.85
Lysine [5]	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Taurine [6]	0.00	0.00	0.00	0.00	0.00	0.00	0.00
DL-Methionine [7]	0.75	0.75	0.75	0.75	0.75	0.75	0.75
Celufil [8]	8.65	8.15	8.15	7.65	6.70	6.96	5.27
Soybean oil	3.45	3.45	3.40	3.35	3.25	3.30	3.15
Vitamin premix [9]	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Mineral premix [9]	4.00	4.00	4.00	4.00	4.00	4.00	4.00
CMC [8]	2.00	2.00	2.00	2.00	2.00	2.00	2.00
ZD1 Bacteria[10]	0.00	0.00	0.55	1.10	2.15	4.30	8.60
Commercial PHB/PHA[11]	0.00	0.50	0.00	0.00	0.00	0.00	0.00
Analyzed Proximate Composition (%)							
Dry Matter	86.9	91.2	89.4	91.6	92.2	90.5	85.7
Crude Protein	35.1	35.1	35.4	35.2	34.8	36.0	36.6
Crude Lipid	5.69	5.30	5.80	6.00	5.52	6.00	6.29
Ash	9.04	8.91	8.89	9.18	9.65	10.7	12.8

Abbreviations: SBM: soybean meal; PHB: Polyhydroxybutyrate; FM: Fishmeal; CMC: Carboxymethyl cellulose

[1] ProFine F, DuPont Nutrition & Health

[2]Omega Protein Corporation, Abbeville, LO

[3]Producers Cooperative Association, Bryan, TX

[4]Rangen, Angleton, TX

[5] ADM Animal Nutrition

[6] TCI Chemicals, Portland, OR

[7]Ajinomoto North America Inc., Itasca, IL

[8]MP Biomedicals, Solon, OH

[9]Same as in Moon and Gatlin III

[10]Chu Biotechnology Lab, Texas A&M University Department of Civil Engineering, College Station, TX

[11]Goodfellow Corporation, Coraopolis, PA, USA

2.4 Diet Formulation and Experimental Design – Hybrid striped bass feeding trial

Isonitrogenous and isolipidic diets for juvenile hybrid striped bass (feeding trial 2) were formulated to contain approximately 40% crude protein and 10% lipid. Similar to feeding trial 1, the basal diet was formulated without PHB, while five experimental diets were supplemented with the PHB synthesizing ZD1 ingredient in place of cellulose to provide PHB at either 0.125, 0.25, 0.5, 1.0, and 2.0% of dry weight (Table 2). In addition, a separate basal diet was supplemented with 0.5% by weight of the afore mentioned commercial PHB ingredient. Experimental diets were then produced and analyzed identically as previously described for feeding trial 1.

Juvenile hybrid striped bass were obtained from Keo Fish Farms (Keo, Arkansas) and acclimated and hardened for 1 week in 30, 38-L aquaria set up as a recirculating aquaculture system while fed a commercial diet (Purina Animal Nutrition, LLC., Arden Hills, MN) containing 42% crude protein. Similar to trial 1, fish were then accurately weighed as groups of 15 fish per aquarium (5.5 g initial weight ± 0.14 SEM), and evenly distributed in each of the 30 aquaria to have similar initial weights. Each diet was randomly assigned to sextuplicate groups of juvenile HSB and fed twice daily their assigned diet at a set percentage of body weight to approach satiation without overfeeding throughout the 8-week feeding trial. Weight gain was monitored weekly by group weighing all fish in each aquarium and adjusting rations equally among diets based on observed feeding behavior to prevent accumulation of uneaten feed in the recirculating aquaculture system. Water quality was analyzed twice a week, and parameters were kept within ranges suitable for HSB including the following: Temperature (˚C): 28.1 ± 0.22; O_2 (mg L^{-1}): 7.11 ± 0.91; TNN

(mg L^{-1}): 0.15 ± 0.13; TAN (mg L^{-1}): 0.23 ± 0.14; Salinity (ppt): 3.10 ± 0.35; pH: 8.02 ± 0.20 (average ± standard deviation).

Table 2: Diet formulation of the experimental diets containing graded levels (g/100g) of PHB from lyophilized *Zobellella denitrificans* or a purified PHB ingredient fed to hybrid striped bass juveniles (feeding trial 2) for 8 weeks.

Ingredients	Basal	Commercial PHB Ref.	ZD1 PHB 0.125	ZD1 PHB 0.25	ZD1 PHB 0.5	ZD1 PHB 1.0	ZD1 PHB 2.0
Soy protein concentrate [1]	17.60	17.60	17.60	17.60	17.60	17.60	17.60
Menhaden fishmeal [2]	12.70	12.70	12.70	12.70	12.70	12.70	12.70
Dehulled soybean meal [3]	38.25	38.25	38.25	38.25	38.25	38.25	38.25
Wheat flour [4]	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lysine [5]	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Glycine [6]	1.00	1.00	1.00	1.00	1.00	1.00	1.00
DL-Methionine [7]	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Celufil [8]	6.11	5.61	5.88	5.65	5.19	4.27	2.43
Menhaden fish oil[2]	7.10	7.10	7.10	7.10	7.10	7.10	7.10
Vitamin premix [9]	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Mineral premix [9]	4.00	4.00	4.00	4.00	4.00	4.00	4.00
CMC [8]	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Dextrinzed corn starch[8]	7.24	7.24	7.24	7.24	7.24	7.24	7.24
ZD1 Bacteria[10]	0.00	0.00	0.23	0.46	0.92	1.84	3.68
Commercial PHB/PHA[11]	0.00	0.50	0.00	0.00	0.00	0.00	0.00
Analyzed Proximate Composition (%)							
Dry Matter	92.5		93.9	93.9	9.27	92.8	91.6
Crude Protein	41.0		41.7	41.8	41.9	41.9	42.1
Crude Lipid	8.07		8.95	8.96	9.00	8.82	8.96
Ash	10.1		10.1	10.2	10.6	11.4	13.2

Abbreviations: SBM: soybean meal; PHB: Polyhydroxybutyrate; FM: Fishmeal; CMC: Carboxymethyl cellulose

[1] ProFine F, DuPont Nutrition & Health

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[3]Producers cooperative association, Bryan, TX

[4]Rangen, Angleton, TX

[5] ADM Animal Nutrition

[6] TCI Chemicals, Portland, OR

[7]Ajinomoto North America Inc., Itasca, IL

[8]MP Biomedicals, Solon, OH

[9]Same as in Moon and Gatlin III

[10]Chu Biotechnology Lab, Texas A&M University Department of Civil Engineering, College Station, TX

[11]Goodfellow Corporation, Coraopolis, PA, USA

2.5 Diet Formulation and Experimental Design – Red drum feeding trial

For feeding trial 3, five isonitrogenous and isolipidic diets were formulated from a practical basal diet to contain 40% CP and 10% lipid. From the basal diet, four experimental diets were supplemented with a commercial PHB product at 0.125, 0.25, 0.5, and 1.0% of dry-diet weight (Table 3). Experimental diets were then produced and analyzed according to identical methods described in feeding trial 1.

Table 3: Diet formulation of the experimental diets containing graded levels (g/100g) of a commercially purified PHB ingredient fed to red drum juveniles (Feeding trial 3) for 8 weeks.

Ingredients	Basal	Com. PHB 0.125	Com. PHB 0.25	Com. PHB 0.5	Com. PHB 1.0
Soy Protein Concentrate [1]	17.80	17.80	17.80	17.80	17.80
Menhaden Fishmeal [2]	16.70	16.70	16.70	16.70	16.70
Dehulled soybean meal [3]	39.80	39.80	39.80	39.80	39.80
Taurine [6]	1.00	1.00	1.00	1.00	1.00
Lysine [4]	0.50	0.50	0.50	0.50	0.50
Glycine [5]	1.00	1.00	1.00	1.00	1.00
DL-Methionine [6]	0.75	0.75	0.75	0.75	0.75
Celufil [7]	1.03	0.9	0.78	0.53	0.03
Menhaden Fish oil[2]	8.42	8.42	8.42	8.42	8.42
Vitamin premix [8]	3.00	3.00	3.00	3.00	3.00

Table 3 Continued

Ingredients	Basal	Com. PHB 0.125	Com. PHB 0.25	Com. PHB 0.5	Com. PHB 1.0
Mineral premix [8]	4.00	4.00	4.00	4.00	4.00
CMC [7]	2.00	2.00	2.00	2.00	2.00
Dextrinzed Corn Starch[7]	4.00	4.00	4.00	4.00	4.00
Commercial PHB[9]	0.00	0.13	0.25	0.50	1.00
Analyzed Proximate Composition (%)					
Dry Matter	86.1	89.6	91.0	90.6	89.6
Crude Protein	47.5	46.6	46.9	47.0	47.0
Crude Lipid	12.0	11.8	12.2	11.1	10.9
Ash	10.5	10.4	10.5	10.5	10.4

Abbreviations: SBM: soybean meal; PHB: Polyhydroxybutyrate; FM: Fishmeal; CMC: Carboxymethyl cellulose

[1] ProFine F, DuPont Nutrition & Health

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[4] ADM Animal Nutrition

[5] TCI Chemicals, Portland, OR

[6]Ajinomoto North America Inc., Itasca, IL

[7]MP Biomedicals, Solon, OH

[8]Same as in Moon and Gatlin III

[9]Goodfellow Corporation, Coraopolis, PA

Juvenile red drum for feeding trial 3 were obtained from Texas Parks and Wildlife Department's Sea Center Texas Hatchery (Lake Jackson, TX) and acclimated for 1 week in 30, 38-L aquaria set up as a recirculating aquaculture system while fed a commercial diet (Rangen, Angelton, TX) containing 42% crude protein. Red drum were then accurately weighed as groups of 15 fish per aquarium, and evenly distributed in each of 25 aquaria to have similar initial weights (4.4 g initial weight ± 0.20 SEM). Each diet was randomly assigned to five replicate groups of

juvenile red drum and fed twice daily at a set percentage of body weight to approach satiation without overfeeding throughout the duration of the 8-week feeding trial. Once again, weight gain was monitored weekly by group weighing all fish in each aquarium and adjusting rations equally among diets based on observed feeding behavior to prevent accumulation of uneaten feed in the recirculating aquaculture system. Water quality was analyzed twice a week, and parameters were kept within ranges suitable for red drum as follows: Temperature (˚C): 26.6 ± 0.93; O_2 (mg L^{-1}): 6.08 ± 0.91; TNN (mg L^{-1}): 0.02 ± 0.01; TAN (mg L^{-1}): 0.11 ± 0.06; Salinity (ppt): 3.72 ± 0.51; pH: 7.76 ± 0.22 (average ± standard deviation).

2.6 Sample Collection and Analysis

Sampling procedures were similar for all three feeding trials. Prior to the start of each feeding trial, an initial sample of 20 fish was randomly selected and stored at -20° C for whole-body proximate composition analysis and computation of protein efficiency ratio based on samples obtained from each aquarium at trial termination. After 8 weeks of feeding the experimental diets, six fish were randomly selected from each aquarium with three anaesthetized with tricaine methanesulfonate (MS-222; Western Chemical, Ferndale, Washington) using a concentration of 100 mg L^{-1}, and the remaining three fish per aquarium were killed using an overdose (300 mg L^{-1}) of MS-222. Those euthanized via an overdose of MS-222 were ground into homogeneous composite samples and analyzed for proximate composition along with initial fish and diet samples according to AOAC (2005) procedures. Briefly, dry matter was assessed by drying samples overnight at 110° C in an oven and homogenized to produce a finely ground sample that was used for the remainder of the analyses. Crude protein was measured by a LECO (St. Joseph, MI, USA) 828 Nitrogen and

Protein Determinator using a sample weight of approximately 0.1 g. Lipid content was then determined on re-hydrated samples by chloroform-methanol extraction, as described by Folch et al. (1957), while ash and organic matter was assessed by subjecting the samples to 650° C for 3.5 h in a combustion furnace.

Blood was collected via heparinized syringes from the caudal peduncle vasculature of three anaesthetized fish per aquarium prior to euthanization with MS-222 at 300 mg L^{-1}. Collected whole blood was aliquoted into 2-mL Eppendorf tubes and used for quantifying blood neutrophil oxidative radical production using NBT as previously described. Remaining blood samples were centrifuged at 3,000 x *g* for plasma separation and stored at -80°C for additional immunological assays such as determination of total plasma protein and immunoglobulins concentration, lysozyme activity, and antiprotease activity to further assess immune function (Ellis, 1990; Yamamoto et al., 2020). Those fish were then ventrally dissected to remove liver and intraperitoneal fat for computation of hepatosomatic index (HSI) and intraperitoneal fat (IPF) ratio, respectively, along with fillet collection to determine muscle yield, as previously described by Rossi Jr. et al. (2015).

Growth performance and body condition indices were computed in all feeding trials as follows:

Percentage weight gain ([g final weight − initial weight/g initial weight] × 100)

Feed efficiency (g dry feed offered/g weight gain)

Protein conversion efficiency ([(final body wt. (g) x final body protein (%)) - (initial body wt. (g) x initial body protein (%))] /protein intake (g)] × 100)

Survival ([#surviving fish / initial stocking density] × 100)

Intraperitoneal fat (IPF) ratio (100 x g IPF weight /g body weight)

Hepatosomatic index (HSI) (100 x g liver weight /g body weight)

Muscle ratio (100 x g fillet weight /g body weight).

2.7 Bacterial Challenge

Following feeding trial 1, the remaining Nile tilapia fed the reference and experimental diets of 1.0 and 2.0% PHB inclusion were maintained on their respective dietary treatments while an LD_{50} dose of virulent *Streptococcus iniae* (obtained from Louisiana State University, Baton Rouge, LA) bacteria was determined using tilapia of similar size. For the LD_{50} determination and subsequent disease challenge, *Streptococcus iniae* bacteria were incubated overnight at 27°C in BHI broth. The bacterial suspension was then washed three times using phosphate buffered saline (PBS), centrifuged at 2,000 x *g* for 10 minutes, and then resuspended in 25 ml of autoclaved PBS. The washed suspension was then diluted to an absorbance of 1.1 at 620 nm to yield a final concentration of 7.0×10^{11} colony-forming units (CFU)/ml (Yamamoto et al. 2018). Mortality was observed for a 14-day duration and recorded to determine survival based on treatment. To confirm cause of death by *S. iniae* infection, head kidneys were isolated from recent mortalities and incubated on tilapia blood infused BHI agar plates.

After the LD_{50} dose was determined, a total of 45 fish per dietary treatment were injected intraperitoneally with 0.1 ml of the predetermined LD_{50} dose and then transferred to separate concrete vats fashioned as a flow-through system receiving a flow of well water at 4.5 L/min. Injected fish were separated in three flow-through vats by dietary treatment and then further divided into groups of 15 fish in each of three replicate baskets measuring approximately 40

cm in diameter. An airstone was placed close to each basket to ensure dissolved oxygen within each basket remained close to saturation.

2.8 Statistical Analysis

All data obtained in the *in vitro* assays and comparative feeding trial were subjected to linear and quadratic regression using JMP Pro 15 software (SAS Institute Cary, NC) to assess significant effects of dietary PHB on growth parameters, immune responses, and condition indices of juvenile Nile tilapia with $\alpha = 0.05$. The model of best fit was chosen based on the lesser p value. Variance homogeneity was verified by the Brown-Forsythe test for all statistical models, while normality was assessed by the Shapiro-Wilk test. If a quadratic relationship was observed, a broken-line linear regression was used to assess the optimal dietary PHB inclusion level using SAS 9.4 software (SAS Institute Cary, NC). Broken-line linear regression was performed as previously described by Portz et al., (2000) to determine the break point associated with each model. The estimate used to determine the breakpoint was determined by visualization using a histogram. In addition to visual histogram breakpoint estimate determination, student's t-test was used to compare means bases on a connecting letters report to best estimate the inclusion percentage of PHB nearest the breakpoint.

3. RESULTS

3.1 In Vitro Assays

It was clearly evident that 3HB successfully inhibited growth of *Aeromonas hydrophila* at a concentration of 50 mM in both pH environments. However, a greater capacity was evident in the acidic medium.

3.1.1 Nile Tilapia In Vitro Results

Isolated Nile tilapia derived leukocytes cultured in graded doses of 3HB exhibited significant positive linear and quadratic relationships when respiratory burst was analyzed (Figures 1 & 2). Both intra- and extracellular superoxide anion production showed a greater adjusted R^2 value and therefore, quadratic regression was used as the selected model for these analyses. However, phagocytic index was not significant when head-kidney derived leukocytes were cultured in media containing graded doses of 3HB (P=0.6 linear and P=0.3 quadratic). Similarly, phagocytic activity also was not significant for leukocytes when analyzed via linear and quadratic regression, (P=0.09 and P=0.16, respectively).

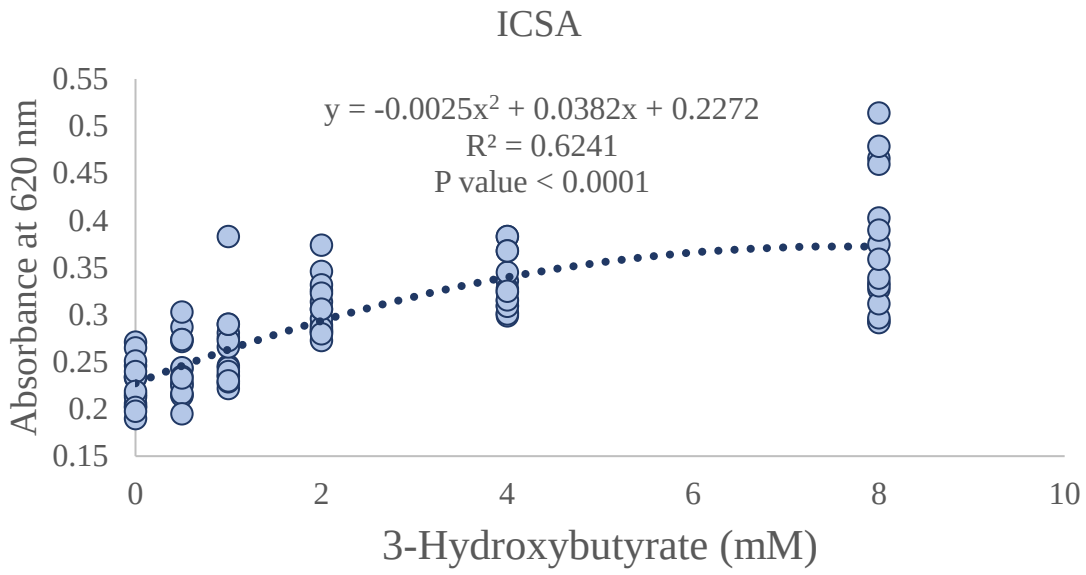

Figure 1. Intracellular superoxide anion production (abs. at 620 nm) of tilapia head kidney-derived macrophages with graded doses of 3-hydroxybuturate.

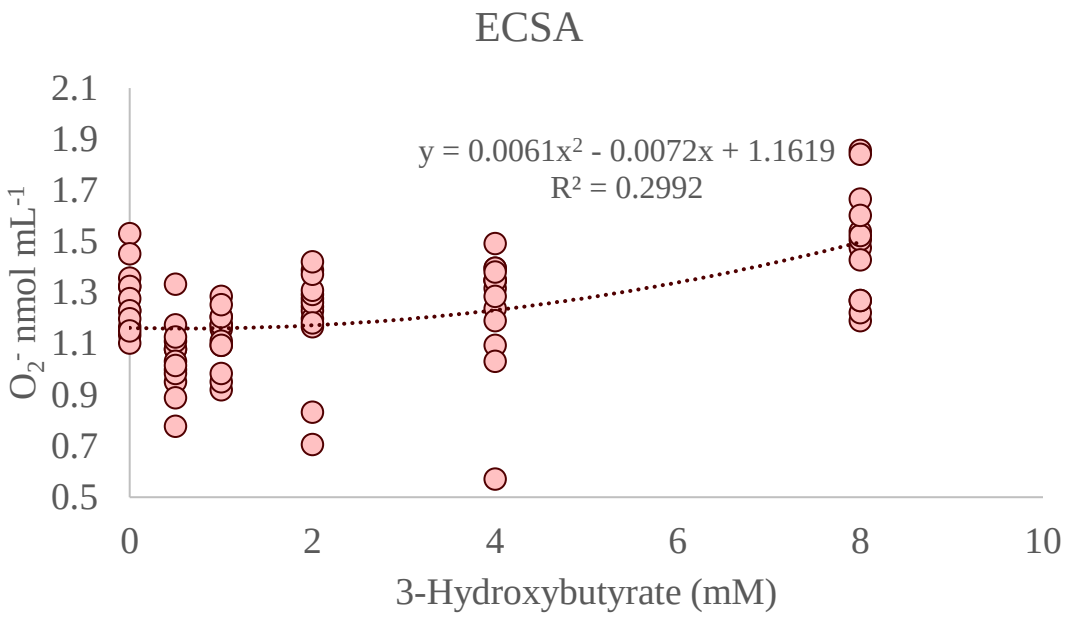

Figure 2. Extracellular superoxide anion concentration (O_{2^-} nmol/well) of tilapia head kidney-derived macrophages with graded doses of 3-hydroxybuturate.

3.1.2 Hybrid Striped Bass In Vitro Results

Surprisingly, isolated HSB-derived leukocytes cultured in graded doses of 3HB were significantly ($P<0.05$) immunosuppressed. Cultured cells had reduced intra- and extracellular superoxide anion production/concentration (Figures 3 & 4) and bactericidal capabilities with increasing concentrations of 3HB (Figure 5). In contrast to Nile tilapia cells, HSB leukocytes cultured in identical conditions were negatively influenced by the addition of 3HB, suggesting a species-specific tolerance for PHB and its derivatives.

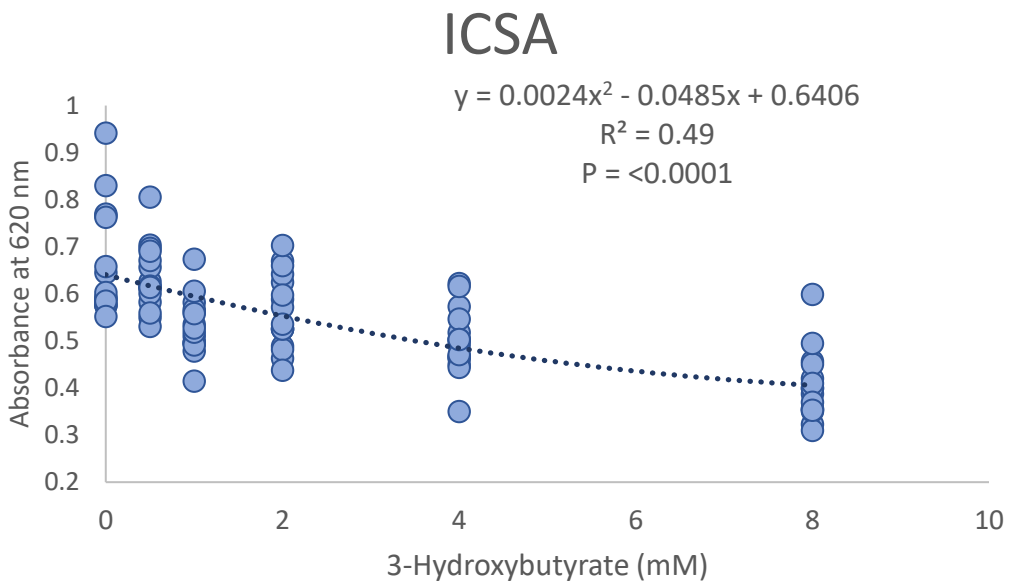

Figure 3. Intracellular superoxide anion production (abs. at 620 nm) of HSB head kidney-derived macrophages with graded doses of 3-hydroxybuturate.

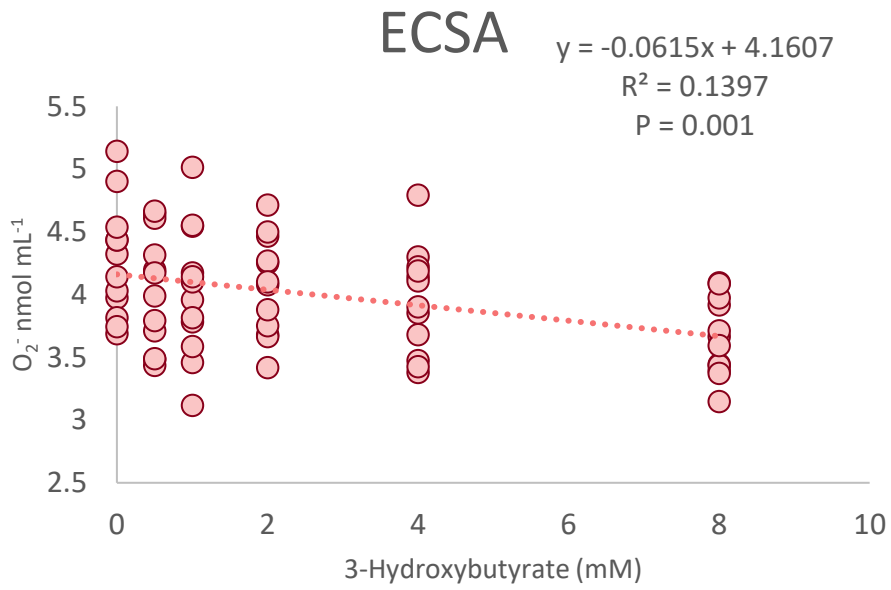

Figure 4. Extracellular superoxide anion concentration (O_{2}- nmol/well) of HSB head kidney-derived macrophages with graded doses of 3-hydroxybuturate.

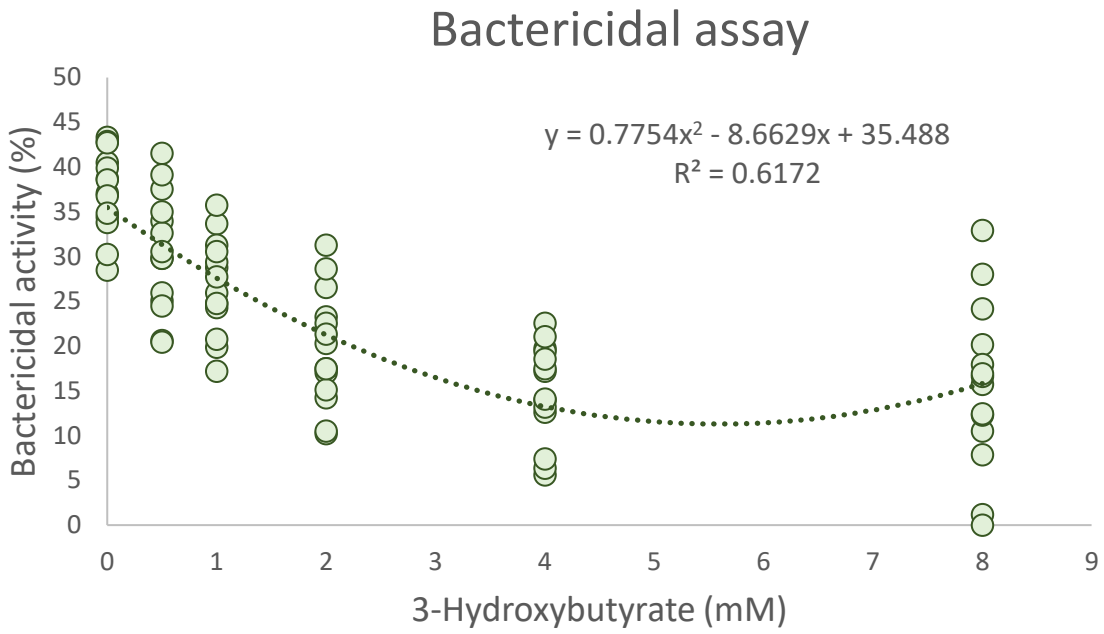

Figure 5. Bactericidal capacity (%) of HSB head kidney-derived macrophages with graded doses of 3-hydroxybuturate.

3.2 In Vivo Feeding Trials

3.2.1 Nile Tilapia Feeding Trial

For feeding trial 1, juvenile Nile tilapia grew rapidly over the 8-week period and did not experience any mortality. Significant improvements in weight gain responses were observed in fish fed diets supplemented with increasing levels of PHB-producing ZD1 bacteria (Table 4). Namely, percentage weight gain and feed efficiency (FE) were quadratically correlated ($P<0.0001$) when ZD1 was supplemented at a higher percentage. To assess any additive effects of unfractionated ZD1 bacterial inclusion in the diets, one way ANOVA was used to compare means of fish fed the basal diet, commercial PHB supplemented diet, and the corresponding 0.5% PHB diet. As analyzed by Tukey HSD, the connecting letters report

revealed a significant increase in percentage weight gain and FE in Nile tilapia fed the 0.5% PHB supplemented diet. However, no significance was shown in percentage weight gain and FE between fish fed the commercial PHB supplemented diet and 0.5% PHB experimental diet (Figure 6). Also, HSI appeared to be weakly correlated linearly with increasing ZD1 supplementation. Other body condition indices of IPF ratio and muscle yield were not significantly affected by dietary PHB similar to NBT oxygen free-radical species production. Whole-body proximate analysis revealed no significant differences for fish fed increasing levels of ZD1 (Table 5). Based on the quadratic relationship of percentage weight gain and FE, a broken-line linear regression was performed to establish the optimal inclusion level of PHB for Nile tilapia. Broken-line regression analysis estimated the optimal inclusion of PHB for percentage gain and FE to be 0.99 (Figure 7) and 0.76% (Figure 8), respectively. As such, 0.99% was determined as the overall optimal inclusion level of dietary PHB for Nile tilapia.

Table 4. Growth performance and condition indices of juvenile Nile tilapia fed experimental diets for 8 weeks.

Diet	**Weight Gain (%)**	**FE**	**HSI (%)**	**IPF (%)**	**Muscle Yield (%)**	**Blood Neutrophil Oxidative Radical Production (ABS at 545 nm)**
Basal	896	0.81	1.12	0.045	25.8	0.293
0.125	1015	0.85	1.42	0.035	27.3	0.312
0.25	1172	0.93	1.18	0.090	27.2	0.321
0.5	1168	0.93	1.41	0.013	27.3	0.331
1.0	1492	1.01	1.47	0.013	26.7	0.327
2.0	1469	1.03	1.55	0.025	27.5	0.308
PSE	90.1	0.033	0.124	0.026	0.856	0.015
Linear Regression P value	**0.0001**	**0.0001**	**0.027**	0.31	0.44	0.82

Table 4 Continued

Diet	Weight Gain (%)	FE	HSI (%)	IPF (%)	Muscle Yield (%)	Blood Neutrophil Oxidative Radical Production (ABS at 545 nm)
Adj. R^2	0.47	0.47	0.17	0.003	-0.02	-0.04
Quadratic Regression P value	**<0.0001**	**<0.0001**	0.07	0.44	0.72	0.17
Adj. R^2	0.59	0.58	0.15	-0.01	-0.06	0.08

Abbreviations: PSE: Pooled Standard Error; Adj. R^2: Adjusted R^2; ABS: Absorbance.

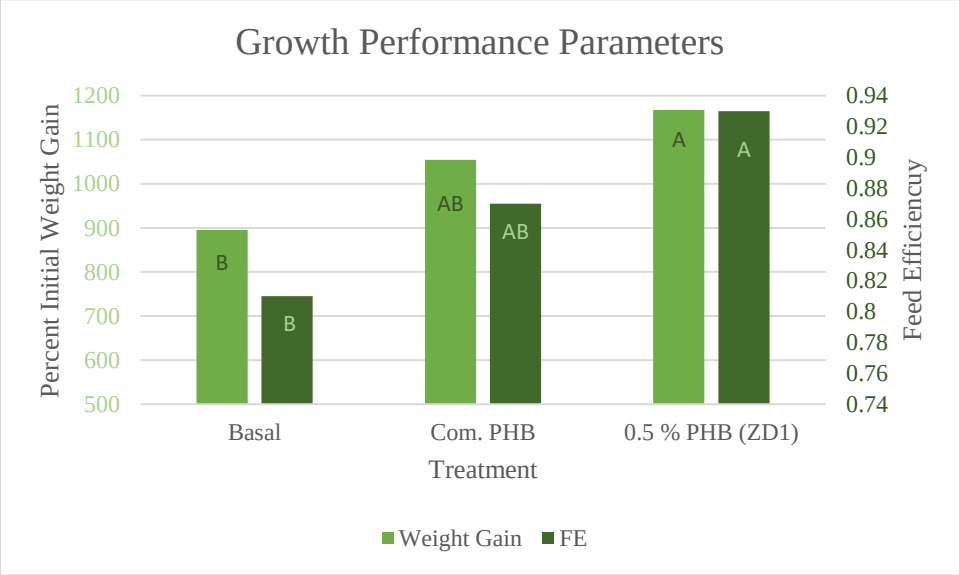

Figure 6. Comparison of a supplemented commercial PHB ingredient compared to the corresponding 0.5% PHB experimental treatment regarding percentage weight gain and FE.

Table 5. Whole-body proximate composition of juvenile Nile tilapia fed experimental diets for 8 weeks (g 100 g^{-1} of fresh body weight unless otherwise noted).

Diet	Moisture	Protein	Lipid	Ash	PCE (%)
Basal	76.4	15.5	5.04	3.54	36
0.125	76.3	15.4	4.97	3.58	38.4
0.25	77.9	14.9	4.09	3.4	35.4

Table 5 Continued

Diet	Moisture	Protein	Lipid	Ash	PCE (%)
0.5	76.6	15.2	4.77	3.33	39.8
1	77.5	14.9	4.13	3.5	39.9
2	76.2	15.3	5.19	3.43	42.1
PSE	0.576	0.224	0.556	0.097	1.40
Linear Regression					
P value	0.66	0.76	0.73	0.57	**0.003**
Adj. R2	-0.04	-0.04	-0.04	-0.03	0.31
Quadratic Regression					
P value	0.33	0.20	0.38	0.69	**0.01**
Adj. R2	0.01	0.06	0.002	-0.06	0.30

Abbreviations: PSE: Pooled Standard Error; Adj. R^2: Adjusted R^2.

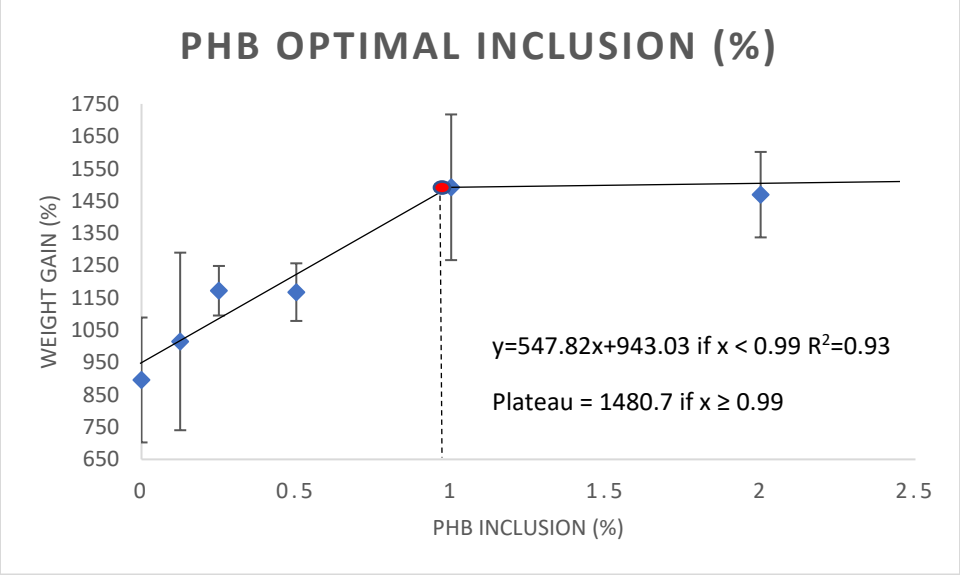

Figure 7. Broken-line linear regression model of percentage weight gain for juvenile Nile tilapia (means ± SEM, n=4).

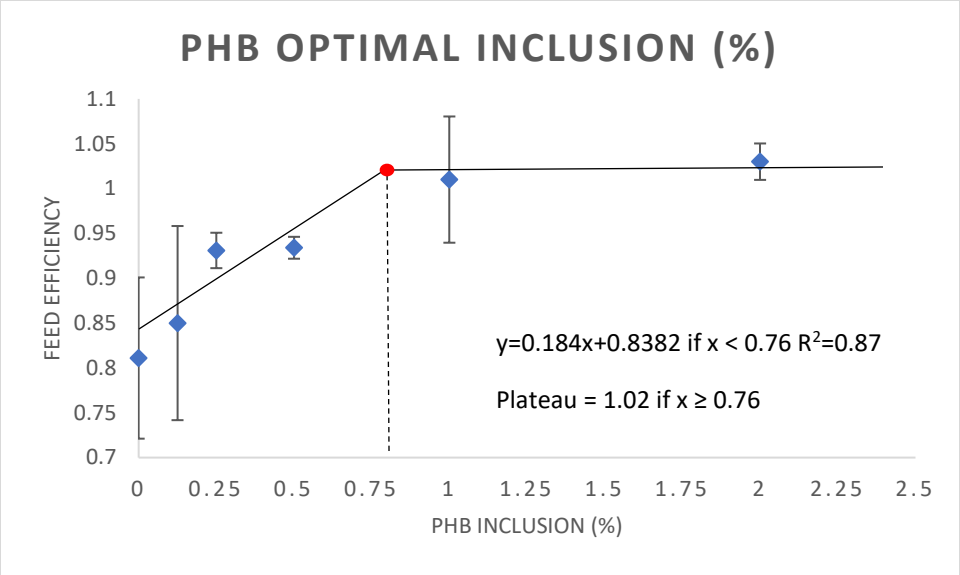

Figure 8. Broken-line linear regression model of feed efficiency for juvenile Nile tilapia (means ± SEM, n=4).

3.3 Nile Tilapia Bacterial Challenge

Survival percentages of exposed tilapia via IP injection to a standardized dose of *Streptococcus iniae*, were 73% in the reference group, 48% in the 1% PHB group, and 50% in the 2.0% PHB group 21 days after exposure (Figure 9). Also, NBT oxygen free-radical production of the disease-exposed fish revealed no significance (P=0.14) when analyzed via one-way ANOVA.

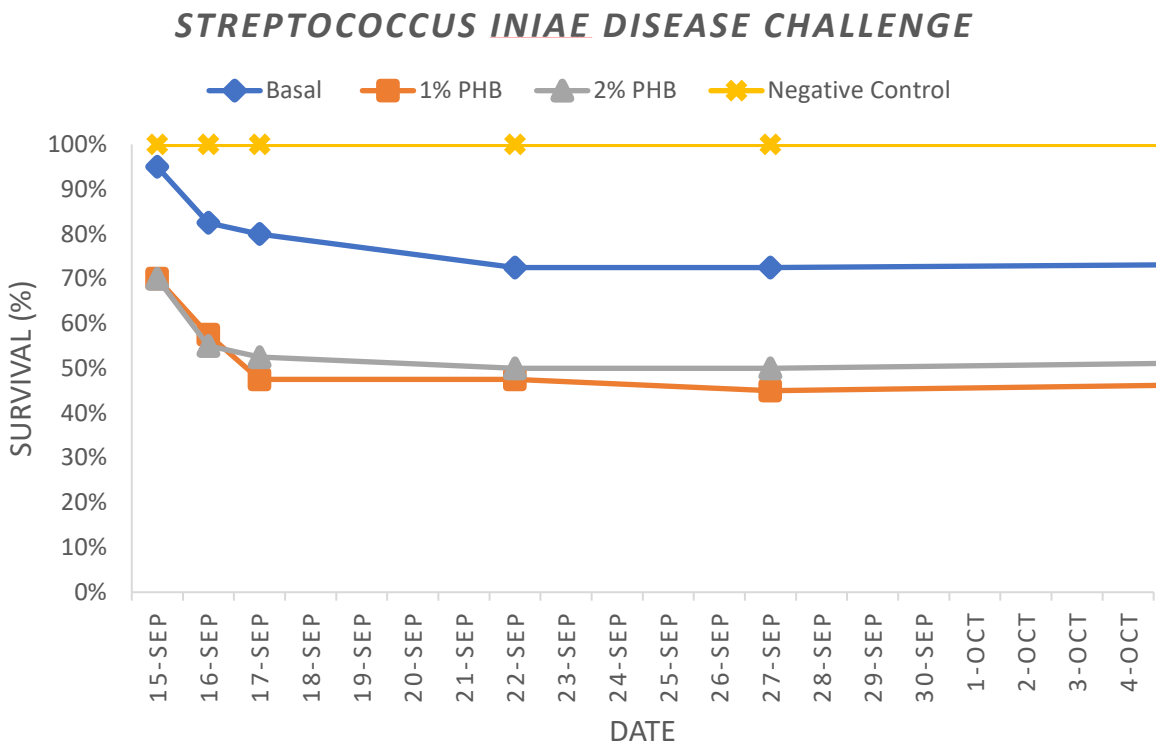

Figure 9. Survival rates (%) of Nile tilapia intraperitoneally injected with *Streptococcus iniae* bacteria observed over 21 days.

3.2.2 HSB Feeding Trial

For feeding trial 2, HSB grew rapidly and increased their initial weight by approximately 700% over the 8-week period. They also had high feed efficiency values and no mortalities were observed in any of the treatments. However, HSB exhibited very limited responses when ZD1 was added to the diets in increasing concentrations. Although percentage weight gain values were numerically higher than the basal treatment for all experimental groups, it was not significantly different (Table 6). Similarly, FE, HSI, muscle yield, and oxygen free radical production responses were also determined to be nonsignificant ($P>0.05$). The IPF ratio displayed a weak quadratic relationship, but significant nonetheless ($P=0.03$).

Table 6. Growth performance and condition indices of juvenile hybrid striped bass fed experimental diets for 8 weeks.

Diet	Weight Gain (%)	FE	HSI (%)	IPF (%)	Muscle Yield (%)	Blood Neutrophil Oxidative Radical Production (ABS at 545 nm)
Basal	699	0.78	1.74	4.39	34.0	0.363
0.125	764	0.81	1.59	3.68	34.7	0.357
0.25	741	0.80	1.55	3.64	34.0	0.338
0.5	755	0.82	1.63	3.69	33.3	0.384
1.0	762	0.81	1.68	3.52	34.7	0.361
2.0	717	0.79	1.58	3.57	34.6	0.355
PSE	24.03	0.011	0.057	0.171	0.604	0.014
Linear Regression						
P value	0.77	0.81	0.79	0.07	0.46	0.96
Adj. R2	-0.041	-0.043	-0.071	0.106	-0.019	-0.045
Quadratic Regression						
P value	0.19	0.08	0.79	**0.03**	0.73	0.78
Adj. R2	0.062	0.136	-0.071	0.217	-0.063	-0.07

Abbreviations: PSE: Pooled Standard Error; Adj. R^2: Adjusted R^2; ABS: Absorbance.

Whole-body proximate composition of HSB revealed a significant linear trend in terms of crude lipid (P=0.006). However, no other proximate composition analyses, including PCE, proved to be significant (Table 7).

Table 7. Whole-body proximate composition (g/100 g fresh body weight) of juvenile hybrid striped bass fed experimental diets for 8 weeks.

Diet	Moisture	Crude Protein	Crude Lipid	Ash	PCE (%)
Basal	70.6	16.4	8.86	3.81	0.312
0.125	70.7	16.6	8.44	3.96	0.325
0.25	70.2	16.6	8.48	3.83	0.318
0.5	71.3	16.5	8.48	3.67	0.322
1.0	70.6	16.6	8.47	3.82	0.322
2.0	71.5	16.3	7.65	3.79	0.307
PSE	0.34	0.15	0.28	0.12	0.006
Linear Regression					
P value	**0.04**	0.31	**0.006**	0.65	0.21
Adj. R2	0.14	0.004	0.26	-0.04	0.03
Quadratic Regression					
P value	0.13	0.35	**0.02**	0.78	0.1
Adj. R2	0.1	0.009	0.24	-0.07	0.12

Abbreviations: PSE: Pooled Standard Error; Adj. R^2: Adjusted R^2; PCE: Protein Conversion Efficiency.

3.2.3 RD Feeding Trial

In feeding trial 3, juvenile RD exhibited no significant differences in growth parameters, including percentage weight gain and feed efficiency (Table 8). Similarly, IPF ratio, survival percentage, and blood neutrophil oxidative radical production was not significant when fish were fed graded doses of the commercial PHB ingredient. Only muscle yield (%) and HSI were significantly ($P<0.05$) elevated as a quadratic trend in red drum fed increasing levels of PHB.

Table 8. Growth and condition indices of red drum fed diets supplemented with incremental levels of PHB.

Treatment	Weight Gain (%)	FE	Fillet Yield (%)	HSI (%)	IPF (%)	Survival (%)	Blood Neutrophil Oxidative Radical Production (Abs. at 545 nm)
Reference	639.3	0.8	28.1	1.48	0.131	60	0.497
0.125	465.5	0.71	27.4	1.67	0.151	68	0.494
0.25	576.8	0.76	28.2	1.63	0.198	64	0.483
0.5	494.7	0.76	27.8	1.85	0.14	76	0.444
1.0	572.7	0.8	29.6	1.65	0.177	74.7	0.502
PSE	37.8	0.025	0.005	0.073	0.031	0.06	0.03
Linear							
Prob > F	0.82	0.29	**0.02**	0.2	0.5	0.07	0.97
Adj. R^2	-0.041	0.008	0.175	0.029	-0.023	0.096	-0.043
Quadratic							
Prob > F	0.18	0.25	**0.03**	**0.01**	0.77	0.13	0.37
Adj. R^2	0.065	0.039	0.202	0.277	-0.065	0.092	0.003

Abbreviations: PSE: Pooled Standard Error; Adj. R^2: Adjusted R^2; ABS: Absorbance.

Whole-body proximate composition of juvenile red drum (feeding trial 3) including crude protein, lipid, and moisture content was not significantly affected by the dietary treatments (Table 9). Only whole-body ash was quadratically significant ($P<0.05$) for red drum fed graded doses of PHB.

Table 9. Whole-body proximate composition (g 100 g^{-1} fresh body weight) of juvenile red drum fed diets supplemented with incremental PHB.

Treatment	Moisture	Crude Protein	Crude Lipid	Ash
Reference	76.0	17.6	2.98	4.22
0.125	76.3	17.6	2.88	4.20
0.25	75.4	18.0	3.34	4.25
0.5	76.1	17.7	2.93	4.18
1.0	75.9	17.7	3.19	4.07
PSE	0.24	0.20	0.14	0.06
Linear				
Prob > F	0.80	0.77	0.38	**0.03**
Adj. R^2	-0.04	-0.04	-0.008	0.16
Quadratic				
Prob > F	0.91	0.80	0.68	0.06
Adj. R^2	-0.08	-0.07	-0.05	0.15

Abbreviations: PSE: Pooled Standard Error

4. DISCUSSION

The collective knowledge of PHB use in various aquatic species suggests overall beneficial effects on growth, intestinal health, and immunological responses, specifically for Nile tilapia (Situmorang, 2015), rainbow trout (Sahin et al., 2021), red drum (Mendoza Rodriguez et al., 2017), and European sea bass (De Schryver et al., 2010). However, the current study observed species-specific effects of PHB action when evaluated with juvenile Nile tilapia, HSB, and red drum. While Nile tilapia exhibited notable beneficial effects in nearly all measured parameters, red drum and hybrid striped exhibited limited effects on growth. Additionally, *in vitro* analyses using HSB macrophage cells even showed deleterious effects of higher levels of PHA.

In vitro analyses of head-kidney leukocytes derived from Nile tilapia were consistent with previous studies. For example, Ebrahimi et al. (2017) observed increased red and white

blood cell counts, lymphocytes, and granulocytes associated with an increased immune response in tilapia supplemented with 2% of dietary butyrate. Additionally, antiprotease, lysozyme, and peroxidase activities significantly increased in Mozambique tilapia when supplemented stepwise with PHB up to 5% (Suguna et., 2014). These documented increases in innate immune activity are understood to be caused by various responses of the host and pathogen to the PHB molecule during degradation into the short chain fatty acid (SCFA) butyrate (Situmorang, 2015). As described previously, SCFAs not only supply immunological cells with a needed energy substrate for effective bactericidal and reproductive processes, they also lower cytoplasmic pH of pathogenic cells, rendering them less effective in invasion (Koh et al., 2016; Laranja & Bossier, 2020; Ng & Koh, 2017). Beneficial effects of PHB supplementation in omnivorous species of the genus *Oreochromis* are well-documented and supported by the present study.

Other reports of augmented immune responses in carnivorous species similar to those observed in cells of fish with omnivorous feeding habits also have been published. For example, beneficial *in vitro* immunological effects have been reported for European sea bass (De Schryver et al., 2010), rainbow trout (Mirghaed et al., 2019), Siberian sturgeon (Najdegerami et al., 2012), and even red drum (Mendoza Rodriguez et al., 2017). However, to the author's knowledge, the current study is the first to attempt to quantify innate immune responses of leukocytes from HSB incubated in PHB-supplemented culture media. Thus, underlying mechanisms for negative effects on HSB-derived-leukocytes cultured in increased doses of 3HB are unknown and require further study.

In regard to the in vivo trials of the current study, feeding trial 1 with Nile tilapia demonstrated a significant quadratic relationship for percentage weight gain, FE, and protein

conversion efficiency which peaked at 1.0% in addition to a numerically higher muscle yield percentage. With an adjusted R^2 value of approximately 0.60 for percentage weight gain and FE, the data suggests a strong correlation as PHB concentration increased up to 2.0%. Similarly, Rodriguez-Estrada (2020) found significantly higher weight gain, specific growth rate, and FE of Nile tilapia when PHA was supplemented at 0.5 and 1.0% of diet compared to a practical reference diet. Additionally, Situmorang et al. (2016) noted, although insignificant, numerically higher percentage weight gain of Nile tilapia fed diets supplemented with PHB at 0.5, 2.5, and 5.0% compared to a reference diet. These similar beneficial results using comparable dietary inclusion levels of PHB/PHA validate the approximate 500% growth response increase in tilapia fed dietary PHB at 1.0 and 2.0% in feeding trial 1 of this study, which is the first to the author's knowledge to establish a defined optimal dietary inclusion level of PHB at 0.99% based on broken-line regression. Although the afore mentioned studies did not statistically analyze PHB and butyrate supplementation of Nile tilapia to determine an optimal inclusion level, the positive effects on production performance were consistently observed across similar dietary concentrations as previously noted.

It is notable that in the current study, an increase in dietary PHB inclusion was correlated with a significant linear increase in HSI of juvenile Nile tilapia, potentially resulting in fatty liver disease, however, proximate composition analysis of fish liver per dietary treatment is necessary to speculate further. While ingredients high in lipid content can induce increased growth and energy utilization, increased lipid uptake can deposit in the liver, causing mortality and reduction in health and immune responses under chronic exposure (Artmann et al., 2008). While hepatic activity and histology was not evaluated in feeding trial 1, fatty liver could have been a complicating factor in the bacterial challenge results of feeding trial 1 explaining

increased growth but reduced survival of fish fed the PHB-supplemented diets when exposed to *Streptococcus iniae*.

The survival percentage results of the present study's bacterial challenge using Nile tilapia injected with a *Streptococcus iniae* bacterial serum is contradictory to not only past experiments, but the *in vitro* and growth performance results observed in the present trial. Jesus et al. (2019b), observed a reduction in mortality (35% reduced to 15%) when Nile tilapia fed dietary sodium butyrate (0.5%) were exposed to virulent *Aeromonas hydrophila*. Situmorang et al. (2016) also observed reduced mortality (70% reduced to 50%) when Nile tilapia larvae were fed PHB-enriched *Artemia* nauplii and exposed to virulent *Edwardsiella ictaluri*. The current trials decrease in mortality in tilapia fed the basal diet (27%) compared with those fed diets supplemented with 1.0 (52%) and 2.0% (50%) PHB are difficult to explain. The author speculates that this negative effect was derived from chronic exposure to the high amount of ZD1 bacteria nucleotides, creating an overstimulation of the immune system that was not present until the addition of a significant stressor, namely the injection of virulent bacteria (Johansen et al., 2006). A negative effect was not observed in the feeding trial due to the pristine water quality and limited stress due to husbandry practices.

In contrast to feeding trial 1, growth performance parameters of HSB and red drum in feeding trials 2 and 3, respectively, were largely unaffected by dietary supplementation of PHB. Only muscle yield for feeding trial 3 was linearly correlated with increased dietary PHB levels of any experiment. The quadratic increase of HSI for feeding trial 3 also could potentially be linked to fatty liver syndrome, as hypothesized in feeding trial 1, however, more hepatic indices would be needed to confirm this assumption. Because of the understudied nature of PHB supplementation with HSB and red drum in particular, very few studies were

found in the literature to corroborate the findings in feeding trials 2 and 3. Further research is warranted to more fully explore the effects of PHB supplementation with these species. Nonetheless, Mendoza Rodriguez et al. (2017) determined a significantly negative effect of red drum weight gain and FE when fed a commercial PHB product which was supplemented at 2.0% of diet. Additionally, rainbow trout (a fish of similar carnivorous feeding behavior and morphology to red drum and HSB) showed no significant growth response when fed diets supplemented with PHB up to 5.0% (Sahin et al., 2020). However, De Schryver et al. (2010), in one of the earliest feeding trials evaluating PHB in the diet of carnivorous species, reported significant increases in weight gain and feed efficiency of European sea bass fed diets with PHB supplemented at 2.5 and 5.0% by weight, compared to those fed the basal diet. Although species-specific differences in metabolic utilization of dietary PHB in terms of growth performance and condition indices have been reported in the literature and observed in the present feeding trials, some of the differences may be related to experimental conditions including initial fish size and trial duration as well as the chemical form and purity of the PHB supplemented in the diet.

Whole-body proximate composition of fish fed diets supplemented with graded levels of PHB in all feeding trials of the present study exhibited limited responses. Nile tilapia, HSB, and red drum in the current feeding trials exhibited no significant responses to PHA supplementation regarding whole-body moisture and crude protein composition, while whole-body crude lipid exhibited a significant negative linear trend only in HSB. Additionally, whole-body ash content of juvenile red drum exhibited a significant negative linearly trend in response to dietary PHA supplementation in feeding trial 3. A possible explanation for decreased whole-body lipid content in response to increasing PHB in the diet could be from

lipolytic effects of cortisol secretion from prolonged immunostimulation possibly due to high levels of dietary nucleotides present in the ZD1 ingredient (Davis et al., 1985; Sunny et al., 2002). However, the findings of a decreasing linear correlation in HSB lipid content is somewhat contradictive of what one might hypothesize. As PHB is degraded in the intestine, SCFAs are produced and available for enterocyte uptake, as indicated by decreased pH in the intestine in European sea bass (De Schryver et al., 2010). Increased SCFA abundance in the intestine would likely lead to increased bioaccumulation of whole-body lipid, however, as indicated by De Schryver et al. (2010), SCFAs also act as an energy substrate for enterocytes and immune-related cells. Nonetheless, if PHB was serving as an energy substrate for HSB enterocytes, one might see a compensatory effect of increased whole-body protein as usual amino acid substrates (i.e., glutamine, glutamate, and asparagine) (Wu, 2013) would be conserved. Ultimately, this was not observed in the current study using HSB, further illuminating a need to more fully characterize the metabolism of different forms of PHB in the diet.

Feeding trials 1 and 2 of the current study utilized whole-cell ZD1 bacteria as a source of PHB to simulate realistic commercial application of a practical feed additive due to the high resource expense of the purification process (Asiri and Chu, 2020). The specific concentrations of PHB in the ZD1 were quantified to ensure accurate supplementation of specific levels of PHB in the experimental diets. However, a potential drawback of the current studies' experimental design was the use of unfractionated ZD1 bacteria instead of a purified PHB molecule which was used in feeding trial 3. Although the commercially purified PHB treatment was not found to be statistically significant to the experimental treatment of equal PHB concentration (Figure 7), percentage weight gain and FE were numerically higher in Nile

tilapia fed the 0.5% ZD1 derived PHB experimental diet. Feed ingredients containing large quantities of bacterial cells are typically rich in nucleotides (e.g., as much as 20% in ethanol yeast products) and other natural compounds such as β-glucans (de Cruz et al., 2020). It is well understood that nucleotides may provide immunostimulatory and gastrointestinal benefits, as well as positively affect growth of most fish species studied of date (Li and Gatlin, 2006). Ridha and Azad (2012) observed significant increases in immuno-competence and overall growth of Nile tilapia when fed a nucleotide-rich probiotic, which was also confirmed by Yamashita et al. (2017). The overall health benefits of PHB may be similar to that of nucleotides, thus limiting definitive conclusions on the mechanistic actions of PHB for increased growth and health as seen in feeding trial 1 with Nile tilapia. Chitosan is another molecule in which positive modulation of the immune response has been observed in Nile tilapia (Abu-Elala et al., 2015; Ibrahim et al., 2021) as well as other species. While the author acknowledges potential conflicting experimental factors from the chitosan-based chemical flocculation of ZD1 bacteria in the current study, logistical limitations during the flocculation process necessitated the use of chitosan. Albeit a clear beneficial response was observed in the current study with Nile tilapia, future research must include purified PHB to potentially differentiate responses to PHB, chitosan and nucleotides.

Additionally, chronic ingestion of whole-bacterial-cell nucleotides has been documented to produce overstimulation of the innate and adaptive immune responses leading to decreased muscle yields and elevated cortisol levels, as well as up regulation of certain stress-related cytokines and gene expression of tumor necrosis factor-alpha (TNFα) (Gause and Trushenski, 2011; Johansen et al., 2006). Experiments completed in this laboratory indicate an optimal level of purified nucleotides at 0.5% of diet for HSB but reduction in fish health at higher

levels (1.5 and 2.0%) (de Cruz et al., 2020). Although HSB did not show reduced growth parameters as PHB and ZD1 content increased, consideration must be given in future research if ZD1 inclusion levels should be increased further. Once again, purification and extraction of PHB apart from ZD1 bacteria should be assessed in future feeding trials to confirm such hypotheses.

Also noteworthy, observed differentiation of physical and chemical properties of the PHB molecule have been documented. Pradhan et al. (2018) found that PHB produced by two bacterial organisms, *Bacillus megaterium* and *Cupriavidus necator*, created a PHB molecule that was less crystalline in nature, as well as more resistant to thermal degradation and denaturing compared to a commonly synthesized PHB molecule. Thus, this molecule appears to have varying chemical characteristics depending on the organism/method of derivation. Ultimately, these chemical and physical differences in the molecule could induce differences in relative growth performances between studies as evidenced between the current study and the study conducted by Mendoza Rodriguez et al. (2017). While the current study utilized a more natural and palatable powder form of the ZD1/PHB/chitosan mix, the 2017 study conducted by Mendoza Rodriguez et al. used a commercially synthesized beaded plastic form that could have negatively influenced the palatability of the diet, creating an externality that is not directly correlated to the PHB molecule itself. As such, the physical and chemical properties of the PHB molecule, as well as the dietary delivery method, must be taken into consideration in future research.

Due to the Covid-19 pandemic, digesta DNA extraction followed by intestinal microbiota next generation sequencing (NGS) was delayed but is currently ongoing at the USDA/ARS Southern Plains Laboratory in collaboration with Dr. Michael Hume. Therefore, the author

cannot comment yet on the efficacy of PHB to influence gut microbiota alterations and/or intestinal health of Nile tilapia and HSB. However, current knowledge of the PHB molecule suggests the potential for improved intestinal health (reduction in enteritis) and modification of intestinal bacterial communities as reported in Nile tilapia (Addam et al., 2019; Jesus et al., 2019) when organic acid blends (0.5%) similar to PHB were supplemented in the diet. Positive responses reported in that study included increased villi height and goblet cell prevalence as well as increased survival of the experimental groups over a control. Additionally, Jesus et al. (2019) observed increased villi width, length, and surface area in Nile tilapia fed diets supplemented at 0.5% with sodium butyrate, a derivative of PHB, which resulted in increased diet digestibility presumably due to improved intestinal health. Dietary PHB supplementation also was found to increase intestinal microbiota diversity and enterocyte health in the more carnivorous European sea bass (Busti et al., 2020; De Schryver et al., 2010), presenting evidence for similar potential modifications in HSB and red drum. Based on increased percentage weight gain and FE of Nile tilapia in feeding trial 1 brought about potentially by increased absorption and intestinal health, the author speculates a positive alteration of intestinal microbe communities will be observed once the NGS analysis is completed. Intestinal microbiota diversity metrices and changes in metabolic function utilizing PICRUSt2 software will be completed as soon as possible.

PHB is a molecule that offers attractive benefits to aquatic organisms as a dietary supplement, as seen in the current study (feeding trial 1). However, the PHB molecule and ZD1 bacteria have the potential to revolutionize current RAS systems, offering a biodegradable bacterial molecule/organism that has both nitrifying properties in the water column (Zhang et al., 2014) and immunostimulatory and potential growth benefits for fish

complete in one system (Asiri and Chu, 2020). Thus, PHB offers many potential applications for future use in the aquaculture industry.

5. CONCLUSION AND SUMMARY

In conclusion, species-specific responses were observed for PHB supplementation to the diet of juvenile Nile tilapia, HSB, and red drum when growth responses, condition indices, whole-body proximate composition, and immunological assays were measured. For feeding trial 1, Nile tilapia, a hardy omnivorous species, experienced increased weight gain and feed efficiency responses with an optimal dietary inclusion level of PHB at 0.99% of diet. Separate *in vitro* immunological assays with leukocytes isolated from head kidney tissues also showed positive responses in oxidative radical production to increasing doses of PHA. However, Nile tilapia exposed to a standardized dose of *Streptococcus iniae* via injection after the feeding trial did not show increased resistance to the pathogen. In fact, fish fed diets supplemented with PHB at 1.0 and 2.0% of diet had reduced survival compared to fish fed the basal diet. On the other hand, HSB and red drum exhibited limited improvements in production performance when PHB was supplemented in the diet up to 2.0% by weight. Coupled with limited growth responses, HSB showed deleterious effects on innate immune response when analyzed via specified *in vitro* procedures. Ultimately, the author can conclude that PHB addition to the diets of Nile tilapia proved to be beneficial; however, more research is warranted to validate the potential benefits of supplementing PHB in the diets of HSB and red drum.

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