AQUA-D-21-0600.R2

Effects of butyrate, propionate, and their combination *in vitro*, and the impacts of their supplementation in high-plant-protein diets to the production performance, innate immune responses, and intestinal microbiota of red drum (*Sciaenops ocellatus*)

Fernando Y. Yamamoto[a,b*], Caitlin E. Older[c,1], Michael E. Hume[d], Aline Rodrigues Hoffmann[c], Delbert M. Gatlin III[a*]

[a] Department of Wildlife and Fisheries Sciences, Texas A&M University System, College Station, TX

[b] National Council for Scientific and Technological Development (CNPq), Brasilia, Brazil

[c] Department of Veterinary Pathobiology, College of Veterinary Medicine & Biomedical Sciences, Texas A&M University, College Station, TX

[d] USDA, ARS, SPARC, Food and Safety Research Unit, College Station, TX

[1] Current address: Texas A&M Veterinary Medical Diagnostic Laboratory, College Station, TX

[*]Corresponding authors: 534 John Kimbrough Blvd., Wildlife, Fisheries and Ecological Sciences, TAMUS, College Station, TX, 77843-2258. E-mail: d-gatlin@tamu.edu and yamamotofy@tamu.edu; yamamotofy@gmail.com

Abbreviations: Anaerobic dilution solution (ADS); ANOSIM: Analysis of similarities; B + P: Butyrate and propionate; CMC: Carboxymethyl cellulose; DGGE: Denaturing gradient gel electrophoresis; FE: Feed efficiency; HA: Hemolytic activity; HSI: Hepatosomatic index; Ig: Immunoglobulin; IPF: Intraperitoneal fat; LDA: Linear discriminant analysis; LEfSe: Linear discriminant analysis effect size; MIC: Minimum inhibitory concentration; NGS: Next generation sequencing; NBT: Nitroblue tetrazolium; OA: Organic acids; OTUs: Operational taxonomic units; PCE: Protein conversion efficiency; PCoA: Principal coordinate analysis; PD: Phylogenetic diversity; PSE: Pooled standard error; ROS: Reactive oxygen species; TP: Total protein

ABSTRACT

The objective of this study was to investigate possible effects of sodium butyrate and sodium propionate individually, and in combination, against common pathogenic bacteria and intestinal microbiota *in vitro*, and when supplemented in high-plant-protein diets for the carnivorous red drum. These organic acids (OA) presented synergistic effects when inhibiting *Aeromonas hydrophila* and *Streptococcus agalactiae*. Additionally, red drum intestinal microbiota were incubated under anaerobic conditions *in vitro* with feed-broth media preparations containing the OA. The microbial communities proved to be mildly affected *in vitro* by the OA, with the butyrate and propionate treatments having 90% similarity with the basal diet when analyzed by denaturing gradient gel electrophoresis. Next-generation sequencing revealed a higher relative abundance of the order Clostridiales and class Clostridia compared to the control diet. For the *in vivo* comparative feeding trial, the OA were supplemented at either 0 or 5 g kg^{-1} of butyrate, propionate, or their combination (5 g kg^{-1} of each OA). Five tanks containing 18 juvenile red drum initially weighing ~4.5 g/fish were fed each diet for 8 weeks after which production performance, whole-body proximate composition, whole-blood and plasma immunological responses, as well as intestinal microbial community profiles were evaluated. Data were analyzed as a mixed model, having a 2×2 factorial design (absence or presence of butyrate and propionate as main factors) and the disposition of the aquaria was used as statistical block. Fish fed diets containing propionate had slightly impaired growth performance, and red drum fed diets with butyrate presented slightly impaired protein conversion efficiency, while dietary butyrate reduced the production of reactive oxygen species in whole-blood compared to fish fed the control diet. Fish fed diets supplemented with butyrate presented a higher relative abundance of *Cetobacterium* spp. suggesting a potential beneficial effect of this additive. The predicted metabolic functions of the intestinal microbiome using PICRUSt2 were most affected by dietary butyrate, which resulted in communities predicted to have enrichment of the cobalamin biosynthesis related pathway. However, supplementation of butyrate with propionate resulted in a higher relative abundance of bacterial taxa associated with intestinal dysbiosis.

27 Keywords: sodium butyrate, sodium propionate, organic acids, digesta microbiota, high-plant-protein
28 diets
29

1. Introduction

Aquaculture is a sector of the food animal protein industry that is expected to contribute substantially to food safety in 2050 (Béné et al., 2015). Within the different species of farmed aquatic organisms, the largest and fastest growing fraction is that of fed aquaculture, *i.e.*, aquatic organisms that rely on the input of formulated diets. As such, along with this growth will be a surge in demand of aquafeeds (Hua et al., 2019). Currently, feed production worldwide for aquaculture is estimated to range between 41-58 million ton yearly (Alltech, 2020; Tacon, 2020), and it is predicted to increase by 15-22 million ton by 2025 (Tacon, 2020). In order to supply raw materials to meet this volume increase, it is paramount to seek sustainable feed ingredients for fish diets and optimize the usage of ingredients that are currently available. Soybean meal (SBM), the most abundant plant protein ingredient to be used in animal feeds, comprising over 70% of seed meal production worldwide, and totaling 236 million metric tons per year (AAS, 2020). The relatively low prices of dehulled SBM, when compared to animal-derived protein ingredients, is an attractive feature for feed formulators. However, the level of inclusion of dehulled SBM in fish diets can be constrained by the adverse effects caused by its anti-nutritional factors, especially for carnivorous fish species (Krogdahl et al., 2010; Zhou et al., 2018). Several studies reported an array of inflammatory responses elicited in various fish species fed diets containing SBM, and which are characterized by enteritis, infiltration of inflammatory cells to the submucosa and lamina propria, reduction of brush-border enzyme activities, loss of mucosal fold architecture, and increased permeability within the intestinal cells (Krogdahl et al., 2010). These structural changes in the intestine caused by dietary SBM often compromise intestinal immune responses (Trushenski, 2015), and disrupt the intestinal microbiota (Ringø et al., 2016).

The gastrointestinal tract of farmed fish is one of the main portals of entry of pathogens while continuously being exposed to a variety of opportunistic pathogens and toxins that can naturally happen in microorganism-rich culture water (Salinas and Parra, 2015). The combination of these environmental conditions and the adverse effects of dietary SBM can predispose cultured carnivorous fish to be more

vulnerable to enteric infections (H. Zhang et al., 2020). Hence, it is of utmost importance to develop nutritional strategies to overcome these environmental challenges and promote fish health and intestinal homeostasis. Among the feed additives that may have beneficial effects on the gastrointestinal tract, organic acids (OA) and their salts have been extensively tested as alternatives to antibiotics for terrestrial animals. These natural compounds are formed during digestion by bacterial fermentation of complex carbohydrates (and to a lesser extent nondigested proteins), with the primary metabolites in the fish gut being acetate, propionate, and butyrate (Hamer et al., 2008; Sauer et al., 2008; Tran et al., 2020).

Butyrate and propionate have received increasing attention as feed additives, not only for their ability to inhibit proliferation of enteric pathogenic bacteria, but also because of their relative stability when chelated with cations (*e.g.*, sodium, calcium, or potassium) during the manufacturing processes in bio-digestors. The chelation methods yield salt crystals, which can reduce losses through volatilization when incorporated in diets, thereby improving the delivery of OA to the intestines. Several studies have reported beneficial effects of dietary supplementation of sodium butyrate, as it can serve as an energy substrate to intestinal epithelial cells, inhibit enteric inflammation, and improve homeostasis of the intestinal microbiota (Hamer et al., 2008). On the other hand, propionic acid, which is reported to be the most commonly used OA in animal feeds (Ng and Koh, 2017), has not been evaluated as thoroughly as butyrate for aquatic animals. Nevertheless, dietary sodium propionate has been reported to increase growth performance and to reduce *Vibrio* spp. counts in the intestines of Pacific white shrimp (*Litopenaeus vannamei*) (Silva et al., 2015, 2016) and European seabass (*Dicentracrchus labrax*) (Wassef et al., 2020), and improve survivability of silver catfish (*Rhamdia quelen*) against *Aeromonas hydrophila* challenge (Pereira et al., 2018).

Red drum (*Sciaenops ocellatus*) is an important euryhaline sciaenid species for aquaculture that is endemic from the southern Gulf of Mexico and Atlantic Ocean reaching northward to the coast of Massachusetts (USA) (Matlock, 1987). In the early 1980s, commercial overfishing of red drum prompted efforts for researching aquaculture practices with this species for restocking wild populations as well as for seafood production. In captivity, this stenophagous fish can transition relatively well to manufactured

dry diets, and at juvenile stages, can consume diets containing high levels of SBM without compromising growth performance or apparent health. Nevertheless, additional investigations are necessary to improve SBM usage by carnivorous teleost fish, like the red drum, and OA supplementation may potentially compensate the negative effects of high SBM diets by enhancing intestinal health. Therefore, the objective of this study was two-fold: 1) to evaluate inhibitory effects of butyrate, propionate, and their combination against two common pathogenic bacteria present in aquaculture, and to evaluate if the supplementation of these OA would affect the intestinal microbiota of red drum *in vitro*; and, 2) to evaluate in red drum the effects of dietary supplementation of butyrate, propionate, and their combination in high-plant-protein-based diets on growth performance, immune responses and their impact on the intestinal bacterial microbiota as assessed by next-generation sequencing (NGS) of the V4 region of the 16S rRNA gene.

2. Material and Methods

2.1 Minimum inhibitory concentration (MIC)

The antimicrobial concentrations of sodium butyrate (cat# AC26319-1000, Acros Organics, Fair Lawn, NJ), sodium propionate (cat# AC14901-0010, Acros Organics), and their combination were evaluated at two different pH levels (6 and 7) to determine the minimum concentrations capable of inhibiting growth of *Streptococcus agalactiae* (03-ARS-BZ-TN-06) and *Aeromonas hydrophila* (ML-10-51K). These two different pH levels were selected to evaluate if the inhibitory capacity of the OA were more prevalent under acidic pH (Defoirdt et al., 2018). Bacterial strains were isolated and identified by USDA-ARS (Auburn, AL), and were kindly donated to this investigation by Dr. Craig Shoemaker. *Streptococcus agalactiae* was cultured in Brain Heart Infusion Broth (cat# 53286, Sigma-Aldrich, Co, St. Louis, MO), and *Aeromonas hydrophila* was cultured in Tryptone Soy Broth (cat# 22092, Sigma-Aldrich), overnight at 27˚C.

Minimum inhibitory concentrations were established for each organic acid, and their combination, in the respective culture broth for each bacterium and at the two different pH levels. Two-fold serial dilutions were performed, starting at 250 mM and yielding 200, 100, 50, 25, and 12.5 mM for each treatment, following the addition of 20 µL for each bacterium inoculum. The respective broths were pipetted (80 µL) to a flat-bottom 96-well microtiter plate with four replicate wells per treatment. Bacterial cultures were diluted to yield a final concentration of 1×10^8 CFU mL^{-1}, and 20 µL were added to each well. For a negative control, no bacteria were added to the media and the positive control consisted of the bacteria incubated only into the respective culture broths. The microtiter plates were incubated for 24 h at 27°C and the MIC was determined as the last dilution to inhibit bacterial growth. After establishing the MIC, the potential synergistic effects between the OA were evaluated at the fixed concentration for all treatments that inhibited at least 50% of the bacterial growth (MIC_{50}).

2.2 Experimental diets, fish, and culture conditions

For the comparative feeding trial, a basal diet was formulated to contain 420 g kg^{-1} of crude protein (CP), 120 g of lipid kg^{-1}, and an estimated digestible energy of 12.85 MJ kg^{-1} (Table 1). Dietary crude protein was formulated to be mostly provided by soybean products (75% of total CP) without compromising the growth performance as previously reported (Rossi et al., 2017) (Table 1). Experimental diets were supplemented with sodium butyrate, sodium propionate, or their combination, at 5 g kg^{-1}, at the expense of cellulose. The basal diet without any organic acid supplementation served as a control. The ingredients of the experimental diets were mixed for 30 min in a V-mixer (Blend Master, Buflovak, NY), and blended in an industrial mixer (A-200 Hobart Meat Grinder, OH) with gradual inclusion of menhaden fish oil and water. The resultant mash was cold-pelleted through a 3-mm die plate and dried at room temperature with forced ventilation for 48 h. The resulting pellets from each treatment were ground and analyzed for proximate composition according to AOAC (2005) procedures (Table 1).

Red drum juveniles were obtained from the Sea Center Texas Marine Aquarium, Fish Hatchery and Nature Center managed by Texas Parks and Wildlife Department (TPWD, Lake Jackson, TX) and

were maintained at the Aquacultural Research and Teaching Facility (ARTF, College Station, TX) prior to commencement of the feeding trial. All procedures performed were approved by the Institutional Animal Care and Use Committee at Texas A&M University (IACUC 2019-0448). Eighteen juvenile red drum with a mean initial weight of 4.5 ± 0.1 g [mean ± standard deviation (SD)] were stocked into each 110-L tank, each tank being considered as an experimental unit, with a total of 20 tanks (n=5 per treatment). A subset of 10 fish from the same cohort were euthanized with an overdose of MS-222 (250 mg L^{-1}, Western Chemical, Ferndale, WA) (Topic Popovic et al., 2012) to compute initial whole-body composition (AOAC, 2005). The rearing system had the experimental units positioned side by side, and the four experimental diets were assigned in a manner that treatments could be blocked according to their disposition. Fish were weighed at the beginning of the feeding trial and subsequently on a weekly basis to adjust the rations according to a percentage of the biomass (initially feeding at 6.5% of biomass and reduced to 2.75% by trial termination). Feeding percentage was adjusted the same for all diets over time in a fashion that fish would be fed close to apparent satiation without overfeeding. A recirculating system setup consisted of a common settling chamber for solids sedimentation, as well as biological and mechanical filters. Aeration was provided continuously to each tank and to the biological filter by air stones connected to a regenerative blower system. The water temperature was conditioned by the ambient air and salinity was adjusted as needed by adding synthetic marine sea salt (Red Sea Salt; Red Sea USA, Houston, TX), aiming to balance hardness and alkalinity. Photoperiod (12h dark:12h light) was provided by fluorescent lights controlled by timers. Water quality was measured three times a week and parameters were maintained within suitable ranges for red drum culture (Neill, 1987) with water replacement or occasional addition of synthetic marine salt. Dissolved oxygen in the water and temperature were monitored using an optical dissolved oxygen meter (ProODO, YSI Inc, OH), pH was measured with a portable pH meter (Pocket Pro pH tester, Hach Company, Loveland, CO), salinity was measured with a portable salinity meter (Pocket Pro Salinity tester, Hach), and total ammonia- and total nitrite-nitrogen were determined photometrically using the test reagents and reading in a Hach spectrophotometer (DR2000, Hach). The average of the water quality parameters throughout the trial were as follows

(average ± SD): Temperature = 26.3 ± 0.6 °C, dissolved oxygen = 6.98 ± 0.70 mg L^{-1}, pH = 8.07 ± 0.25, salinity = 4.36 ± 1.42 g L^{-1}, total ammonia-nitrogen = 0.03 ± 0.04 mg L^{-1}, and total nitrite-nitrogen = 0.02 ± 0.01 mg L^{-1}.

2.3. Sampling procedures

Fish from the feeding trial were sampled on three different occasions. On the day 30, digesta were collected from four fish selected from each experimental unit. Fish were fed to apparent satiation, staggered in 10-min intervals to ensure all tanks would have digesta collected 5 h postprandial by the sampling time. The two biggest and two smallest fish from each tank were selected, euthanized with an overdose of MS-222, individually weighed, externally disinfected with ethanol, and then had their gastrointestinal tracts aseptically excised. The intestinal contents were stripped with forceps under sterile conditions into screw capped microcentrifuge tubes and immediately frozen in liquid nitrogen. Digesta samples were transported in liquid nitrogen and stored at -80°C until further processed. The sum of the weight of individual fish removed per tank was subtracted to adjust the daily rations for the remaining days of that week.

At the end of the 8th week, each experimental unit had all fish counted and group-weighed to calculate production performance responses. Three fish from each tank were separated, anesthetized with MS-222 (100 mg L^{-1}), and blood was collected through the caudal vasculature with heparinized tuberculin syringes. An aliquot of the whole-blood samples for each treatment were tested for respiratory burst of leucocytes as described below. The remaining blood was centrifuged 10,000 × *g* for 10 min for plasma collection, which was distributed into 0.5-mL aliquots into microcentrifuge tubes and stored frozen (-80°C) until further processed. The same group of fish from which blood was collected were euthanized as previously described, were individually weighed and their livers and peritoneal cavity fat were excised and weighed to compute hepatosomatic index (HSI) and intraperitoneal fat (IPF) ratio. In addition, one side of each fish were filleted and skinned to obtain fillet yield. Three additional fish from each tank also were euthanized with MS-222, and stored at -20°C until they were homogenized for whole-body

proximate composition. Production performance parameters and condition indexes were computed as follows:

Weight gain (% of initial weight) = 100 × [(Average weight at the 8^{th} week (g) – average initial weight (g)/ average initial weight (g))]

Feed efficiency (FE) = (Weight at the 8^{th} week – Initial weight)/ dry feed intake

Muscle yield (%) = 100 × {[(fillet muscle weight (g) × 2)/ body weight (g)]}

Viscerosomatic indices (HSI or IPF) (%) = [Liver or IPF weight (g) ÷ body weight (g)] × 100

Protein conversion efficiency (PCE) (%) = {[(Final body weight (g) × Final body protein (%)) – (initial weight (g) × initial body protein (%))] ÷ protein intake (g)} × 100

Survival (%) = 100 × (number of surviving fish/initial number of fish)

The remaining fish were fed the experimental diets for an additional 4 days, and at the 60^{th} day, digesta from four randomly selected fish from each experimental unit were pooled, using the aforementioned feeding and sampling procedures.

2.4 Immune parameters for whole-blood and plasma samples

Respiratory burst was measured following the protocols established by Siwicki et al. (1994) with modifications (Yamamoto et al., 2020). Briefly, a 50-µL aliquot of the collected whole-blood from each fish was incubated with an equal volume of nitroblue tetrazolium solution (NBT, cat# 97061-412, VWR International, Radnor, PA) (2 mg mL^{-1} in phosphate buffer saline (PBS, cat# 97062-732, VWR International)). After 30 min of incubation, the formazan granules were re-suspended with 1mL of dimethyl formamide (cat# 227056, Sigma Aldrich), centrifuged at 3,000 × *g* for 5 min, and the absorbance of the supernatant was read at 545 nm in a spectrophotometer. Hemolytic activity was measured photometrically according to Sutili et al. (2016) with modifications. Red drum plasma (100 µL) was incubated with 25 µL of a diluted tilapia erythrocytes solution (washed three times after collection

with PBS and diluted 1:10). Red drum plasma and tilapia erythrocyte samples were incubated for 2 h at room temperature (25°C) in a 96-well U-shaped microplate. After incubation, 150 µL of ice-cold PBS was pipetted into each well to stop the hemolytic activity of the complement proteins, and plates were centrifuged at 3,000 × *g* for 5 min. Each sample was performed in duplicate, and as a positive control, erythrocytes were incubated with 100 µL of deionized water for total hemolysis. Plasma total protein were measured with Coomassie blue (cat#500-0006, Bio-Rad Laboratories, Hercules, CA) and plasma total immunoglobulin was measured by subtraction after precipitating the diluted samples with 12% polyethylene glycol (PEG, cat# P6667, Sigma Aldrich Co) (Siwicki et al., 1994). The lysozyme activity was determined by the reduction of absorbance (Ellis, 1990) when 20 µL of plasma was incubated with 200 µL of *Micrococcus lysodeikticus* (200 mg L^{-1} of PBS) (cat# M3770, Sigma Aldrich Co) for 5 min.

Lysozyme activity (U mL^{-1}) = [(initial absorbance – final absorbance)/Δ time ÷ 0.001] × 100

2.5 Fish and digesta microbiota *in vitro*

In order to evaluate an intestinal microbiome *in vitro* model previously established for red drum (Burr et al., 2010), a group of 16 red drum juveniles from the same cohort were fed the control diet during 30 days twice a day, and reared concomitantly with the feeding trial, in a separate aquarium (110-L), but in the same recirculating system. On the day 30, fish were fed to satiation, euthanized with the procedures described above, and individually weighed (~52 g). Digesta was aseptically collected 5 h postprandial from four groups, four fish per group, and intestinal contents were pooled in four separate 15-mL sterile polystyrene tubes, and diluted (1:1000) with an anaerobic dilution solution (ADS) as described by Burr et al. (2010). The ADS was selected for being a non-nutritive medium, thus limiting the input of nutrients solely by the experimental diets, and mimicking the digestion and fermentation processes similar to those in the fish during the feeding trial. Approximately 12 mL of each of the four pooled digesta were divided four times in separate 15 mL tubes containing 0.3 g of each experimental diet, in a fashion that all pooled groups were exposed to all experimental diets (n=4). A total of 16 samples were incubated in the

anaerobic atmosphere (5% CO_2, 5% H_2, 80% N_2) for 18 h at 27°C, flash-frozen in liquid nitrogen, and stored at -80°C until DNA extraction.

2.6 DNA isolation, PCR and denaturing gradient gel electrophoresis (DGGE)

Samples were thawed in a water bath at room temperature, and immediately centrifuged at 5,000 × *g* for 10 min. The pellet was separated from the supernatant, and 0.2 g of the pellet was transferred to a sterile DNA free RNA free microcentrifuge tube. The genomic DNA isolation protocol was followed according to the manufacturer's instructions (cat# 51306, QIAamp DNA Mini Kit, Qiagen, Valencia, CA) with minor modifications. The pellet was mixed with 180 µL of lysis buffer (20 mg mL^{-1} lysozyme 20 mM Tris-HCl, pH 8.0; 2 mM EDTA, 1.2% Triton X-100) and incubated in a water bath at 37°C for 30 min. Proteinase K, and 4 µL of RNAse A (100 mg mL^{-1}) were added and samples were incubated in a water bath at 56°C for 30 min, then at 95°C for 15 min. After these steps, isolated DNA was quantified using a Nanodrop One (ThermoFisher, Madinson, WI, USA), and DNA samples were diluted to 50 ng of DNA μL^{-1}. Samples were aliquoted and a subset of the samples were shipped to the University of Minnesota Genomics Center for sequencing.

Another subset of samples were subjected to PCR using universal bacterial primers, flanking the V3 region of 16S rRNA gene (Hume et al., 2003). Primers were mixed with Jump Start RED-Taq Ready Mix (cat# P0982, Sigma Aldrich) according to manufacturer instructions. The PCR was performed according to Hume et al. (2003), and resulting products were verified with 4% agarose gels (cat# G800804, Invitrogen Carlsbad, CA). Then, PCR products from samples of the same treatment were pooled and 5 µL from the pooled sample was mixed with an equal volume of loading buffer 2× (bromophenol 0.05%, xylene cyanol 0.05%, glycerol 70%). From this mixture, 7 µL was loaded onto a polyacrylamide gel (8% v/v acrylamide-bisacrylamide ratio of 37.5:1). Electrophoresis was performed for 17 h at 59°C and 60 V using a DCode Universal Mutation Detection System (Bio-Rad Laboratories), after which the gel was carefully removed and stained with SYBR Green I (1:10,000 dilution) for 15 min and images were digitalized. Comparison of the samples band patterns was assessed using the Dice

262 percentage similarity coefficient (%SC) and dendrograms were constructed using the unweighted pair
263 group with arithmetic averages (UPGMA) method in Gel Compare II 6.6 (Applied Maths, Inc., Austin,
264 TX).
265
266 2.7 Next generation sequencing
267 Extracted DNA samples were used for sequencing on an Illumina MiSeq (Illumina, San Diego,
268 CA) at the University of Minnesota Genomics Center. The V4 region of the 16S rRNA gene was targeted
269 using primers 515F: GTGCCAGCMGCCGCGGTAA and 806R: GGACTACHVGGGTWTCTAAT as
270 previously described by Gohl et al. (2016), but with 30 cycles for amplification. Raw fastqs files are
271 available under the BioProject ID PRJNA 725509 in the NCBI Sequence Read Archive. Primers and
272 adapters were removed from the resulting data using cutadapt. Data were further processed in QIIME2
273 (version 2020.2) (Bolyen et al., 2019) where denoising was performed with DADA2 (Callahan et al.,
274 2016) using all default parameters, and truncating the sequences at 220 nucleotides for forward reads, and
275 199 nucleotides for reverse reads. Taxonomic classification was performed with a scikit-learn classifier
276 (Abraham et al., 2014) containing the 99% observed taxonomic units (OTUs) sequences from the SILVA
277 database release 132 (Quast et al., 2013), trimmed to the 515F/806R sequencing region (Bokulich et al.,
278 2018). For the phylogenetic diversity metrics, sequences were inserted into the SILVA 128 SEPP
279 (Janssen et al., 2018) reference tree. A total of 347,440 sequences were obtained after quality filtering,
280 with an average of 24,287 sequences per sample. Prior to diversity analyses, data were rarefied to 4,490
281 sequences per sample for the *in vitro* data and 1,210 sequences/samples for the *in vivo* data, based on
282 analysis of rarefaction plots. Microbiota sequencing data also were used to predict functional output of the
283 microbiota, using PICRUSt2 (Douglas et al., 2020).
284
285 2.8 Statistical analysis
286 Data from the MIC trial and the feeding trial were analyzed using JMP software (v 14.0, SAS
287 Institute, Cary, NC). The MIC assay was analyzed as a 2 × 2 factorial design, and the feeding trial was

analyzed as a 2 × 2 factorial design mixed with a block design, having the disposition of the aquaria as the statistical blocks. The presence or absence of butyrate and propionate were considered as main factors. If significant differences were detected for the factors ($P<0.05$), a student t-test was performed, and if significant differences were detected by the interaction of the factors, data was subjected to Tukey-HSD test. For the NGS data, alpha diversity was calculated using the Shannon diversity index, Chao1, observed features, Faith's phylogenetic distance and Pielou's evenness metrics. Beta diversity was calculated with the Jaccard, Bray-Curtis, and weighted and unweighted UniFrac metrics. Distance matrices resulting from beta diversity analysis were visualized using Emperor (Vásquez-Baeza et al., 2013) and statistically analyzed using the analysis of similarities (ANOSIM) test available in the vegan (Oksanen et al., 2013) package for R (R Core Development Team, 2019). Testing for differential relative abundance and differences in predicted functional output were performed using the linear discriminant analysis effect size (LEfSe) algorithm (Segata et al., 2011) found on the Huttenhower Lab Galaxy (https://huttenhower.sph.harvard.edu/galaxy/). For LEfSe results, a linear discriminant analysis (LDA) score ($\log_{10}$) > 2.5, and $p < 0.01$ were considered significant.

3. Results

3.1 *In vitro* experiments

A synergistic effect was observed in the MIC assay for butyrate and propionate at pH 6 and 7 against *Aeromonas hydrophila* (Figure 1A). At pH 6, the supplementation of both organic acids had a higher inhibitory capacity than singular additions, followed by butyrate, and lastly the propionate group. A comparable result was observed at pH 7, where both organic acids had a higher inhibitory capacity, when compared to the individual organic acids. However, at this pH, the individual supplementation of butyrate and propionate were not different from each other. A synergistic effect was also observed for inhibiting the proliferation of *Streptococcus agalactiae*, but only at pH 7 (Figure 1B). Butyrate +

propionate had a higher inhibitory capacity than propionate alone, but it was not statistically different from butyrate alone.

Digesta incubated *in vitro* with the experimental diets presented a bacterial community which appeared identical for the propionate, and B + P groups based on the DGGE dendrograms (Figure 2A). The control and butyrate groups also had very similar bacterial populations (91.2% and 92.4% SC, respectively), when compared to the propionate and B + P groups. Microbiota from *in vitro* samples were primarily composed of Firmicutes (average relative abundance = 65%), followed by Fusobacteria (31%), and Proteobacteria (2%) (Figure 3A). Samples incubated with the basal diet presented significantly higher relative abundances of Clostridia (LDA Score = 5.09, P = 0.01), more specifically the family Clostridiales (LDA Score = 5.18, P = 0.01) (Figure 3A), when compared to the other treatments. No significant differences were observed for alpha or beta diversity (Table 2), or for the predicted functions of the microbiome.

3.2 *In vivo* experiment

Final weight and growth performance of juvenile red drum were significantly impaired for fish fed diets supplemented with propionate (Table 3). Feed efficiency values were marginally significant (P = 0.055) for the interaction between butyrate and propionate, with fish fed B + P having lower values. No significant differences in dietary treatments were observed for the body condition indices fillet yield, HSI, or IPFs, as well as for survival. The lipid in whole-body tissues significantly decreased for the fish fed diets supplemented with propionate (Table 4). Protein conversion efficiency also was significantly impaired when fish were fed diets supplemented with butyrate (Table 4). No differences due to dietary treatment were observed for moisture, protein and ash contents of whole-body tissues.

In terms of immunological responses, the production of reactive oxygen species (ROS) measured by the reduction of NBT by the phagocytes in whole-blood was significantly affected by the interaction between butyrate and propionate supplementation (Table 4). Fish fed the control diet had higher production of ROS compared to fish fed diets supplemented with butyrate. No differences were observed

for the lysozyme and hemolytic activities, or for soluble protein and immunoglobulin concentrations in plasma. Nevertheless, it is noteworthy that a reduced P-value for lysozyme activity was observed regarding the interaction between butyrate and propionate, with a higher numerical value for the propionate treatment.

The DGGE dendrograms (Figure 2B and 2C) from digesta samples of red drum fed the various diets suggested that the microbial populations were very similar among treatments. Samples obtained on the day 30 showed that fish fed the control diet harbored microbial communities that appeared to be different from fish fed the diets supplemented with either of the OA. However, on day 60, fish fed the control diet and the diets supplemented with propionate or B + P presented a microbiota likely identical (96.3 and 100% SC, respectively); whereas, fish fed the diets supplemented with butyrate had a microbiota population very similar (90.9% SC) to that of the latter treatments.

NGS data showed that the digesta microbiota from red drum fed the experimental diets was primarily composed of Proteobacteria, as the most abundant phylum (average relative abundance = 36%), followed by Firmicutes (30%), Bacteroidetes (17%), Fusobacteria (5%), and Actinobacteria (5%) (Figure 3B). Fish fed the control diet had higher relative abundance of the order Lactobacillales when compared to the other treatments (Figure 4B). The digesta microbiota of fish fed diets supplemented with butyrate had higher relative abundance of Fusobacteria, specifically *Cetobacterium* spp, and Clostridia, specifically *Paraclostridium* spp. A higher relative abundance of Enterobacteriaceae, an unclassified *Enterobacteriaceae* genus, and Plactomycetales were found in the digesta of fish fed diets supplemented with propionate. And lastly, fish fed diets supplemented with B + P presented higher relative abundances of *Delftia* spp., *Variovorax* spp., *Microbacterium*, and uncultured *Oligoflexaceae*. Thus, supplementation of OA altered the microbial composition of the digesta and also translated to significant differences in the predicted functions of the microbiome (Figure 5). Fish fed diets supplemented with butyrate had more bacteria associated with fermentation of nucleotides to organic acids, degradation of acetylneuraminate, biosynthesis of cyanocobalamin (vitamin B_{12}), degradation of glycerol to butanol, biosynthesis of polyamine, and *de novo* biosynthesis of deoxyribonucleotides. The supplementation of propionate in the

365 diets of red drum resulted in a community enriched with the bacteria associated with fatty acid elongation,
366 TCA cycle, and heme B biosynthesis from glutamate. No significant differences were found for alpha
367 diversity analysis of microbiota from the feeding trial (Table 5). Analysis of beta diversity with three
368 metrics (Bray-Curtis, Jaccard, and Weighted-UniFrac) resulted in significant P-values, but relatively low
369 R values indicating a low dissimilarity in community structure (Supplementary Figure 1).
370
371 4. Discussion
372
373 With the growing concern over the development of antimicrobial resistant bacteria (AMB),
374 stricter regulations for the usage of antibiotics are being implemented for food animal production, but
375 special attention should be given to aquatic species. The possibility of disseminating therapeutics and
376 AMB to the environment is believed to be greater for aquaculture, when various culture systems do not
377 have their effluents properly treated then discharged into rivers, estuaries, or the sea (Defoirdt et al., 2011;
378 Watts et al., 2017). As an alternative to conventional antibiotics, OA are natural fermentation metabolites
379 produced by enteric bacteria that can inhibit the growth of pathogenic bacteria (Defoirdt et al., 2011). It is
380 hypothesized that the antibacterial mechanisms of OA include diffusion of these molecules into the
381 cytoplasm and acidification of the intracellular contents, leading the bacterial cells to exhaustion by ATP
382 depletion from expending energy to export excess protons from the cytoplasm (Ng and Koh, 2017).
383 Additionally, OA also can interfere with and disrupt membrane proteins and membrane structure of
384 bacteria, inhibit electron transport and ATP production, and impede synthesis of macromolecules (Ng
385 and Koh, 2017). These antimicrobial properties combined with the potential roles of dietary OA in
386 maintaining intestinal homeostasis make them promising additives for remediating intestinal issues
387 caused by high inclusion of SBM and preventing harmful bacterial infections.
388 In the *in vitro* study, the growth of *Aeromonas hydrophila* and *Streptococcus agalactiae* in
389 culture media were successfully inhibited by the addition of butyrate, and propionate and predictably,
390 their combination had a synergistic/higher inhibitory property when compared to the addition of

individual OA. The bacteriostatic efficacy of OA depends on the pH of the environment, where in a lower pH, they can remain in the undissociated form and passively diffuse across the bacterial cell membrane. Therefore, it was expected to observe higher inhibitory properties by the OA salts in culture media with pH 6 than media with pH 7. In contrast, no differences were detected for inhibitory capacity of *Streptococcus agalactiae* cultured at a lower pH. Perhaps the acidic environment by itself did not permit this fastidious bacterium to properly proliferate. Butyrate had a superior inhibitory capacity against *Aeromonas hydrophila* when compared to propionate at pH 6, but at a neutral pH this difference could not be detected. Corroborating the present findings, similar bacterial inhibitory results under acidic and neutral pH were observed by Bolivar et al. (2018) and Pereira et al. (2018) when testing butyrate and propionate against *Aeromonas hydrophila*, and Jesus et al. (2019) testing butyrate against *Aeromonas hydrophila* and *Streptococcus agalactiae*. The bacteriostatic properties of propionate, butyrate, and hydroxybutyrate also were reported against different pathogenic *Vibrio* species in a pH-dependent manner (da Silva et al., 2013; Defoirdt et al., 2018), which also may possibly reduce the activity of their virulence factors (Defoirdt et al., 2018).

In vitro intestinal models are an interesting approach to assess the prospective impacts of a given diet or feed additive on the metabolite production and shifts in the core intestinal microbiota (Nissen et al., 2020). An *in vitro* fermentation model, previously established to investigate the effects of a commercial prebiotic on the production of short-chain fatty acids and the modulation of the cultured intestinal gut microbiota (Burr et al., 2008, 2010), was adapted in the present study to evaluate if the addition of butyrate and propionate salts could also modify the microbiota composition without compromising the population richness. The results from the preliminary DGGE analysis appeared to be promising, given that the band patterns obtained from the gels of the *in vitro* and the *in vivo* (day 60) experiments were somewhat comparable, which prompted sending these samples for NGS. However, it was observed from the relative abundance data that these samples were mainly composed of the phyla Firmicutes and Fusobacteria. When comparing these results with the samples from the feeding trial, it appears that the phyla Bacteroidetes, Proteobacteria and Actinobacteria did not thrive in the anaerobic

417 dilution solution, presenting negligible levels of these phyla in the samples, if they were not totally
418 absent. The complexity of the intestinal bacteria community combined with the fastidiousness of some
419 bacteria species can be a constraint for the development of an *in vitro* model to mimic the gut microbiota
420 (Nissen et al., 2020). A striking difference of the model employed with the natural physiological digestion
421 of red drum is that the experimental diets added to the inoculum did not undergo an acidic hydrolysis and
422 protease digestion, which could have limited the availability of amino acids and peptides in the culturing
423 suspension. To our knowledge, this preliminary approach was the first attempt at assessing the bacterial
424 population using NGS of an *in vitro* intestinal model for fish, but adaptations are still needed to better
425 portray the richness of the *in vivo* microbiota. Nevertheless, establishing such model could be a valuable
426 tool for the interdisciplinary field of aquaculture nutrition and microbial ecology research, by providing
427 better insights on interactions between the host bacterial community with different dietary health
428 promoters or pathogenic microorganisms, and possibly predicting changes in the gut microbiota
429 functional pathways. In the present findings, it was observed that the relative abundance of the order
430 Clostridiales and class Clostridia was higher for the control treatment, suggesting that the
431 supplementation of OA either inhibited the abundance of this order and class, or proliferation of other
432 bacteria taxa resulted in a lower relative abundance of this order and class for the other treatments.
433 For this feeding trial, production performance and survival of red drum reached remarkably high
434 values for being fed high-plant-protein diet; however, the supplementation of butyrate and propionate did
435 not improve performance. In fact, the addition of propionate to the diets decreased growth performance,
436 and the supplementation of both OA (B + P) marginally impaired feed efficiency. Similar results were
437 observed for Artic charr (*Salvelinus alpinus*) fed diets containing 100 g kg^{-1} of sodium propionate for 84
438 days in brackish water (Ringø, 1991). The author hypothesized that the depressed growth performance
439 could be attributed to a lower digestibility of lipids and proteins, found in high concentrations in the
440 digesta. Interestingly, the lipid content of whole-body tissues of red drum fed diets with propionate were
441 significantly lower compared to fish fed non-propionate diets. When the IPF ratio values were checked, a
442 numerical trend also was noticed in which fish treated with organic acids having lower values compared

443 to fish fed the control diet. It has been previously reported for mice and swine that dietary propionate can
444 inhibit the synthesis of fatty acids *in vitro* (Delzenne and Kok, 1998), and the expression of hepatic
445 lipogenic genes decreased the concentration of serum lipids (Jiao et al., 2020; Weitkunat et al., 2016).
446 However, it remains unclear whether the low lipid content of the whole-body is correlated with a lower
447 lipid digestibility, or if the lipid metabolism of red drum could have been affected by dietary propionate.
448 Contrasting with the present results, a plethora of studies recently reported enhanced growth
449 performance of fish fed either butyrate or propionate. In those studies, different sources of butyrate were
450 tested (butyric acid or sodium butyrate), and an improved weight gain could be attained with
451 supplementation ranging from 2-10 g kg^{-1} for golden pompano (*Trachinotus ovatus*) (Zhou et al., 2019),
452 barramundi (*Lates calcarifer*) (Aalamifar et al., 2020), and Nile tilapia (*Oreochromis niloticus*) (Jesus et
453 al., 2019). In contrast, calcium or sodium propionate seemed to be required at higher inclusion levels for
454 different fish species, ranging from 5-30 g kg^{-1}, to promote enhanced growth performance of yellowfin
455 seabream (*Acanthopagrus latus*) (Sangari et al., 2020), Caspian white fish (*Rutilus frisii kutum*)
456 (Hoseinifar et al., 2016), silver catfish (*Rhamdia quelen*) (Pereira et al., 2018), and European seabass
457 (*Dicentrarchus labrax*) (Wassef et al., 2020). Superior growth performance was also reported when fish
458 were fed diets containing high inclusion of plant protein ingredients supplemented with sodium butyrate
459 or butyrate glycerides (0.25-20 g kg^{-1}) such as that reported for yellow drum (*Nibea albiflora*) (Wu et al.,
460 2020), black sea bream (*Acanthopagrus schlegelii*) (Volatiana et al., 2020), Asian swamp eel
461 (*Monopterus albus*) (J. Zhang et al., 2020), and turbot (*Scophthalmus maximus*) (Liu et al., 2019), or for
462 yellowfin seabream fed sodium propionate (5 g kg^{-1}) (Sotoudeh et al., 2020). Nonetheless, different from
463 all aforementioned investigations using marine carnivorous species, red drum in the present study were
464 reared under low-salinity conditions, a factor that could have indirectly precluded the beneficial aspects of
465 these additives. Under a hyperosmotic environment, fish are prompted to exert more energy to maintain
466 the osmotic balance (Ern and Esbaugh, 2018), and to ingest seawater to compensate osmotic loss of water
467 across the gills and skin (Evans, 1993). Hence, it is hypothesized that the positive effects from dietary
468 butyrate and propionate could be more evident when culturing carnivorous species in full strength

469 saltwater, where these supplements can serve as metabolic substrates for enterocytes, and/or perhaps
470 inhibit proliferation and exposure of ingested pathogenic bacteria to the intestine, thereby maintaining
471 enteric homeostasis and promoting growth. In support of this hypothesis, no improvements in growth
472 performance were observed for freshwater carnivorous fish fed diets supplemented with butyrate, as seen
473 with rainbow trout (*Oncorhynchus mykiss*) (Gao et al., 2011) or the arapaima (*Arapaima gigas*) (Luz et
474 al., 2019); however, more investigations are recommended to elucidate if there are effects of dietary OA
475 with the culture water salinity.

476 The plasma immune responses of red drum were not significantly affected by the dietary
477 supplementation of sodium butyrate, sodium propionate or B + P. These findings are in agreement with
478 studies evaluating these additives separately (Benedito-Palos et al., 2016; Pereira et al., 2018), but are in
479 conflict with several other reports where dietary OA promoted increased levels of diverse immune-related
480 enzyme activities and proteins in the plasma/serum, or upregulation of genes associated with
481 inflammatory cytokines in the intestine or lymphoid organs of several fish species (Aalamifar et al., 2020;
482 Abd El-Naby et al., 2019; Safari et al., 2017, 2016; Ullah et al., 2020). In the current trial, only a reduced
483 P–value for the interaction between butyrate and propionate was observed for plasma lysozyme activity,
484 suggesting a numerical trend. Although immunostimulation was reported for many teleost species fed
485 diets with OA, the mechanisms of how these molecules trigger the inflammatory cascade and enhance the
486 immunological responses are still not fully understood (Hoseinifar et al., 2017). *In vitro* models for
487 humans also have presented controversial results for different tissues and cell types, suggesting that
488 endogenous butyrate and propionate may stimulate migration of neutrophils to the intestine (Vinolo et al.,
489 2009), and butyrate can upregulate the production of inflammatory cytokines (IL-8 and IL-1β) of the
490 intestinal epithelial cells (Böcker et al., 2003). On the other hand, butyrate downregulated the expression
491 of pro-inflammatory cytokines (IL-6 and IL-12) when intestinal macrophages were exposed to
492 lipopolysaccharides (Chang et al., 2014), and induced the expression of the anti-inflammatory cytokines
493 (Liu et al., 2018). These assertions were translated to fish nutrition studies when graded levels of dietary
494 butyrate increased leukocyte infiltration in the proximal segment of the intestine (J. Zhang et al., 2020),

and diminished the effects of SBM-induced inflammation by decreasing the production of TNF-α, IL-1β and IL-6, and increasing the production of IL-10 and TGF- β1 in the distal intestine (Benedito-Palos et al., 2016; Wu et al., 2020). With the reduced production of reactive oxygen species (ROS) of red drum whole-blood, it is not possible to discern if this was an anti-inflammatory reaction promoted by dietary butyrate, which could have reduced the number of active circulating phagocytes in the blood, or if this reduced number of phagocytic cells was the outcome of an increased chemotaxis of neutrophils to the intestine, as observed in human models (Vinolo et al., 2009), and possibly in the Asian swamp eel (J. Zhang et al., 2020).

The gut microbiota plays an important role in fish nutrition and health by aiding the digestive function and gastric development, maintaining intestinal mucosal barriers, interacting with the immune system, and conferring increased disease resistance (Merrifield et al., 2011; Romero et al., 2014). The high inclusion of SBM in the diets of farmed carnivorous teleost has shown antagonistic effects to intestinal health by promoting enteritis, impairing the intestinal mucosa (H. Zhang et al., 2020), and disrupting the gut microbiota (Bruce et al., 2018; Green et al., 2013). To mitigate these issues, the supplementation of OA in high plant-protein diets for fish has proven to be a promising nutritional strategy due to their ability to restore intestinal microbiota (Piazzon et al., 2017), and enhance the competency of the intestinal structure and mucosal barriers (Liu et al., 2019; Volatiana et al., 2020; Wu et al., 2020; J. Zhang et al., 2020). Further characterization of the red drum gut microbiota by NGS showed communities were mainly comprised of Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, and Fusobacteria (in decreasing order of relative abundance), which is in agreement with what has been reported for other fish species (Yukgehnaish et al., 2020). A similar relative abundance of these phyla also were observed in a previous study from our research group, in which red drum were also fed high-SBM diets and cultured in a low-salinity environment (Rossi et al., 2017).

At the order level, Lactobacillales, which also has been addressed as lactic acid bacteria (LAB), were found to have higher relative abundance in red drum fed the control diet. A higher relative abundance of LAB has been repeatedly reported for different carnivores species consuming diets with

SBM including the Atlantic salmon (*Salmo salar*), rainbow trout, gilthead seabream (*Sparus aurata*), and the olive flounder (*Paralichthys olivaceus*) (Gajardo et al., 2017; Niu et al., 2020; Parma et al., 2016; Reveco et al., 2014). These authors hypothesize that the increases in relative abundance may occur due to the ability of LAB to utilize non-starch polysaccharides from SBM (*e.g.,* raffinose and stachyose). Indeed it is counterintuitive to have this supposedly beneficial taxa increasing under a challenging nutritional condition (Desai et al., 2012), and it is unknown if they are promoting intestinal health as the levels of SBM increases in the experimental diets. Nonetheless, the present findings suggest that dietary supplementation of butyrate, propionate, or B + P in high-SBM diets may have suppressed the proliferation of lactic acid bacteria in the intestine of red drum.

Red drum fed butyrate supplemented diets exhibited a higher relative abundance of the phylum Fusobacteria appears to be directly related down the phylogenetic line to the genus *Cetobacterium* spp.. Previous reports associated *Cetobacterium somerae* with inhibition of common pathogenic bacteria present in aquaculture (Sugita et al., 1996), and with production of vitamin B_{12} in the intestine of freshwater fish (Tsuchiya et al., 2008). The latter can be associated with the predicted function output data, which indicated intestinal communities in red drum fed butyrate had compositions more involved with cobalamin synthesis pathways. This bacterial species appears to be of importance for intestinal nutrition and health, with prevalence in the intestine of wild freshwater carnivorous species (Liu et al., 2016). However, it is noteworthy to mention that *Cetobacterium* spp also was found in the intestine of wild-caught red drum in a marine environment, and it was the second most abundant species in the intestinal microbiota (Givens et al., 2015). A higher relative abundance of the genus *Paraclostridium* was also associated with dietary butyrate, and was reflected at the class level, with higher relative abundance of Clostridia. Interestingly, this finding contradicts results from the *in vitro* study. Regarding the predicted functions of the intestinal microbiome, the relative abundance of *Paraclostridium* spp. appears to be connected to the following pathways: pyruvate fermentation to acetate and lactate, degradation of the N-acetylneuraminate (sialic acid), and glycerol degradation to butanol. It is possible that the synthesis of butanol from glycerol is related to the production of butyric acid, a feature presented in some species from

547 the Clostridiaceae family (Lehmann et al., 2012). Sialic acid is present in the mucin produced by goblet
548 cells, and it is quite intriguing to observe a significant enrichment of communities with predicted function
549 for the degradation for this carbohydrate, given that either commensal or pathogenic bacteria can share
550 this metabolic pathway (Vimr et al., 2004). After bacterial enzymatic degradation, sialic acid can yield
551 pyruvate, which could be used as a fermentation substrate and production of acetate and lactate as
552 metabolites.

553 Higher relative abundance of the genus Unclassified Enterobacteriaceae was observed for the red
554 drum fed diets with propionate, which also was reflected at the order level, with a higher relative
555 abundance of the order Enterobacteriales. Interestingly, dietary propionate affected the predicted
556 functions of the intestinal microbiota, by predicting enrichment of the fatty acid biosynthesis pathway,
557 which could be related to the lower lipid content of the red drum whole-body. Red drum fed diets
558 supplemented with propionate also presented a higher relative abundance of bacteria of the order
559 Planctomycetales, and fish fed B + P had a higher relative abundance of *Delftia* spp. and *Variovorax* spp.
560 These taxa have been associated with disturbed intestinal homeostasis in mosquito fish (*Gambusia affinis*)
561 and grass carp (*Ctenopharyngodon idellus*), where these species were either exposed to a pathogenic
562 bacterium, intestinal inflammation, or to antibiotic treatment (Carlson et al., 2017, 2015; Tran et al.,
563 2018). Therefore, it is possible that the supplementation of propionate and both organic acids in high-
564 plant protein diets could have caused detrimental effects to the red drum intestinal microbiota. Possible
565 pathogenic strains of concern in aquaculture, such as *Aeromonas* spp or *Streptococcus* spp., which can
566 naturally occur in the intestinal microbiota of fish, did not have their relative abundance affected by the
567 experimental diets.

568 Alpha diversity results obtained from the NGS data suggest that supplementation of butyrate,
569 propionate, or B + P did not affect the richness or evenness of the microbial community. Previous studies
570 observed similar results where supplementation of a commercial OA blend (SiloHealth™) to gilthead sea
571 bream (*Sparus aurata*) (Rimoldi et al., 2018) or sodium butyrate (20 mg kg^{-1}) to turbot (Liu et al., 2019)
572 did not significantly affect alpha diversity. On the other hand, gilthead sea bream presented an evident

increase of richness of the intestinal microbiota when fed diets with sodium butyrate (Piazzon et al., 2017). Several intrinsic factors during a feeding trial can modulate the gut microbiota composition and diversity, such as environmental (water quality, diet, and season) and physiological conditions (genetic diversity, and sex) (Romero et al., 2014). In the case of the latter study with gilthead sea bream (Piazzon et al., 2017), the predominance of Vibrionaceae, a family found in the intestine of saltwater fish, could be more susceptible to butyric acid treatment, leading to their reduction and an increase in diversity of the intestinal microbiota. Even though a significant P-value for ANOSIM tests of beta-diversity was observed in the present study with red drum, the low R value suggests community structure was not strongly influenced by OA supplementation. Similar to the present results, Niu et al. (2018) did not find clustering of samples based on dietary treatment for the intestinal microbiome of olive flounder during the juvenile phase; however, they did observe an increasing dispersion of the samples as the life-stage of the fish progressed; thus, it could be hypothesized that if the feeding trial associated with the present study was prolonged or if samples were collected from larger animals, the dissimilarities between the dietary treatments would be more distinct.

In conclusion, sodium butyrate and propionate are potent bacteriostatic molecules, and successfully inhibited the proliferation of the pathogenic bacteria *Aeromonas hydrophila* and *Streptococcus agalactiae in vitro*, and a synergistic inhibition was observed when the OA were combined. Further adaptations of the *in vitro* fermentation model of red drum intestinal microbiota are necessary to better portray the microbial richness as observed in samples collected *in vivo*. Great efforts have been made to diminish the deleterious effects of high SBM inclusion in the diet of red drum but no prominent results concerning production performance were achieved in the present study with the inclusion of OA in high-plant-protein diets for this species. The supplementation of sodium butyrate impacted the intestinal microbiota and predicted functions of the microbiome, possibly in a beneficial way; however, the supplementation of sodium propionate and butyrate with propionate may have disrupted the intestinal microbiota by resulting in communities associated with disease and stress. More research is encouraged to explore further the positive impacts of dietary butyrate in the intestinal structures of red drum, evaluate

599 the effects of this supplement under commercial settings, where water quality would not be as pristine as
600 a recirculating system, and finally to ascertain if the predicted functions of the intestinal microbiota
601 observed in the present study can be physiologically translated to the red drum.
602

603 Declaration of competing interest

604 The authors confirm that there is no conflict of interest associated with the manuscript entitled, "The
605 effects of butyrate, propionate, and their combination *in vitro*, and the impacts of their supplementation in
606 high-plant-protein diets for the production performance, innate immune responses, and intestinal
607 microbiota of red drum (*Sciaenops ocellatus*)".

608

609 Acknowledgements

610 This work was conducted at Texas A&M University Aquacultural Research and Teaching Facility
611 (ARTF) and was partially funded by Texas A&M AgriLife Research. At the time of this study, Fernando
612 Yugo Yamamoto was a Tom Slick Graduate Fellow, sponsored by Texas A&M AgriLife Research, and
613 his doctoral degree was partially funded by the Science Without Boarders' program, sponsored by the
614 Brazilian National Council for Scientific and Technological Development (CNPq 207141/2014-2). The
615 computational resources used to analyze the intestinal microbiome sequencing were provided by Texas
616 A&M High Performance Research Computing (https://hprc.tamu.edu). The authors would like to thank
617 Dr. Craig Shoemaker and USDA-ARS AL for kindly providing the bacteria cultures for the *in vitro* study.
618 The authors also gratefully acknowledge the support provided by Mr. Brian Ray, Mr. Fernando Campero,
619 and Mr. Charles Hernandez for their technical support throughout the feeding trial and laboratorial
620 analyzes. This study was accomplished with the assistance provided by the graduate students of Texas
621 A&M Fish Nutrition Laboratory during the feeding and sampling procedures.

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945 Table 1: Formulation and analyzed proximate composition of the experimental diets used in the *in vitro*

946 trial and the comparative feeding trial. Values are expressed as g per kg^{-1}.

Ingredients (g kg^{-1})	Basal	Butyrate	Propionate	B + P
Menhaden fishmeal[1]	154.0	154.0	154.0	154.0
Soy protein concentrate[2]	178.0	178.0	178.0	178.0
Dehulled soybean meal[3]	361.5	361.5	361.5	361.5
Dextrinized corn starch[4]	40.0	40.0	40.0	40.0
Menhaden oil[1]	92.5	92.5	92.5	92.5
Glycine[4]	10.0	10.0	10.0	10.0
Lysine [5]	10.0	10.0	10.0	10.0
Taurine[4]	10.0	10.0	10.0	10.0
DL-methionine[6]	7.5	7.5	7.5	7.5
CMC[4]	20.0	20.0	20.0	20.0
Cellufill[4]	46.5	41.5	41.5	36.5
Mineral premix[4]	40.0	40.0	40.0	40.0
Vitamin premix[7]	30.0	30.0	30.0	30.0
Sodium butyrate[8]	0.0	5.0	0.0	5.0
Sodium propionate[8]	0.0	0.0	5.0	5.0
Analyzed proximate composition (g kg^{-1})[9]				
Moisture	86.6	86.7	84.8	85.8
Protein	468.1	466.7	461.4	465.1
Lipid	130.6	129.4	136.9	132.8
Ash	103.0	107.6	103.4	105.4

947 [1] Omega Protein Corporation, Abbeville, LO

948 [2] ProFine F. DuPont Nutrition & Biosciences, New Century, KS

949 [3] Producers Cooperative Association, Bryan, TX

950 [4] MP Biomedicals, Solon, OH

951 [5] ADM Animal Nutrition, Quincy, IL

952 [6] Ajinomoto North America Inc., Itasca, IL

953 [7] Same as in Moon & Gatlin III (1991)

954 [8] Acros Organics, Fair Lawn, NJ

955 [9] Values are expressed in dry matter basis, and means of three replicate analysis

956 Abbreviations: CMC; Carboxymethyl cellulose; B + P: Butyrate and Propionate.

957

958 Table 2: Growth performance, condition index, and survival of red drum after 8 weeks of feeding the

959 experimental diets.

	Initial weight (g)	Final weight (g)	Growth performance (%)	FE	Fillet yield (%)	HSI (%)	IPF (%)	Survival (%)
Treatment means[1]								
Control	4.5	53.5	1067.7	1.03	27.7	1.92	0.97	98.5
Butyrate	4.4	53.1	1059.9	1.04	26.9	1.79	0.76	97.1
Propionate	4.4	51.9	1033.6	1.04	27.3	1.76	0.70	100.0
B + P	4.5	50.8	1009.0	1.00	26.1	1.73	0.74	97.1
PSE	0.04	0.7	16.4	0.01	0.5	0.09	0.09	1.3
Main effect means[2]								
Butyrate (g kg^{-1})								
0.0		52.7	1050.3	1.03	27.5	1.84	0.84	99.3
5.0		51.9	1034.5	1.02	26.5	1.76	0.75	97.1
Propionate (g kg^{-1})								
0.0		53.3^{A}	1063.8^{A}	1.03	27.3	1.85	0.86	97.8
5.0		51.4^{B}	1021.3^{B}	1.02	26.7	1.75	0.72	98.6
ANOVA *P values*								
Butyrate		0.34	0.34	0.14	0.07	0.39	0.37	0.14
Propionate		0.02	0.02	0.19	0.26	0.25	0.16	0.61
Butyrate × Propionate		0.61	0.62	0.055	0.65	0.55	0.21	0.61
Block		0.04	0.04	0.04	0.02	0.99	0.66	0.30

960 Different superscript letters are significantly different ($P<0.05$).

961 [1] Values represent means of five replicate tanks (n = 5).

962 [2] Values represent means of ten replicate tanks (n = 10).

963 Abbreviations: B + P: Butyrate and propionate; FE: Feed Efficiency; HSI: Hepatosomatic index; IPF:

964 Intraperitoneal fat; PSE: Pooled standard error.

965

966 Table 3: Whole-body proximate composition and protein conversion efficiency of red drum after 8 weeks

967 of feeding the experimental diets. Values expressed as g kg^{-1}, unless otherwise noted, on wet-basis.

	Moisture	Protein	Lipid	Ash	PCE (%)
Treatment means[1]					
Control	725.5	186.5	27.2	40.5	36.1
Butyrate	728.3	185.6	25.4	40.4	35.5
Propionate	729.5	189.8	23.7	39.3	37.3
B + P	731.9	185.9	23.8	40.2	34.7
PSE	2.7	1.4	1.1	0.6	0.6
Main effect means[2]					
Butyrate (g kg^{-1})					
0.0	727.5	188.2	25.5	39.9	36.7^{A}
5.0	730.1	185.8	24.6	40.3	35.1^{B}
Propionate (g kg^{-1})					
0.0	726.9	186.1	26.3^{A}	40.5	35.8
5.0	730.7	187.9	23.7^{B}	39.8	36.0
ANOVA *P values*					
Butyrate	0.37	0.13	0.47	0.53	0.02
Propionate	0.19	0.24	0.04	0.30	0.75
Butyrate × Propionate	0.94	0.32	0.40	0.47	0.14
Block	0.79	0.85	0.29	0.66	0.15

968 Different superscript letters are significantly different ($P<0.05$).

969 [1] Values represent means of five replicate tanks (n = 5).

970 [2] Values represent means of ten replicate tanks (n = 10).

971 Abbreviations: B + P: Butyrate and Propionate; PCE: Protein conversion efficiency; PSE: Pooled

972 standard error

973

974 Table 4: Immunological responses of whole blood and plasma from red drum fed the experimental diets

975 for 8 weeks.

	NBT	Lysozyme	HA	TP	Total Ig
	(Abs. at 540 nm)	(U mL^{-1})	(%)	(mg mL^{-1})	(mg mL^{-1})
Treatment means[1]					
Control	0.621^{A}	96.6	34.1	21.2	10.2
Butyrate	0.591^{B}	108.9	30.3	21.5	12.0
Propionate	0.596^{AB}	128.9	34.2	20.5	12.9
B + P	0.598^{AB}	82.2	31.3	21.0	12.1
PSE	0.006	14.8	3.3	0.01	0.01
Main effect means[2]					
Butyrate (g kg^{-1})					
0.0	0.608	112.7	34.2	20.8	11.8
5.0	0.595	95.5	30.8	21.2	12.1
Propionate (g kg^{-1})					
0.0	0.606	102.8	32.2	21.3	11.3
5.0	0.597	105.5	32.7	20.7	12.5
ANOVA *P values*					
Butyrate	0.04	0.26	0.33	0.62	0.69
Propionate	0.16	0.85	0.88	0.51	0.09
Butyrate × Propionate	0.02	0.07	0.89	0.89	0.13
Block	0.01	0.01	0.58	0.92	0.06

976 Different superscript letters are significantly different ($P<0.05$).

977 [1] Values represent means of five replicate tanks (n = 5).

978 [2] Values represent means of ten replicate tanks (n = 10).

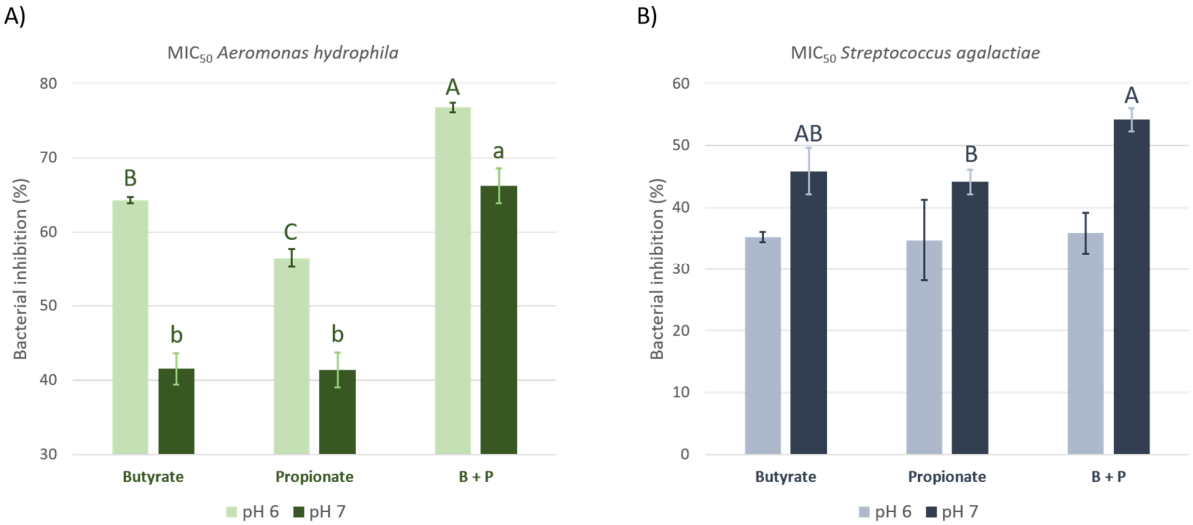
979 Abbreviations: Abs.: Absorbance; B + P: Butyrate and Propionate; NBT: Nitroblue tetrazolium; HA:

980 Hemolytic activity; TP: Total protein; Ig: Immunoglobulin; PSE: Pooled standard error

Table 5: Alpha and beta diversity results of microbiota from red drum digesta evaluated *in vitro* and *in vivo*. Alpha diversity was compared across treatments using Kruskal-Wallis test, and beta diversity was analyzed using analysis of similarities (ANOSIM) on distance matrices. P-values < 0.05 indicate statistically significant results for either test. For ANOSIM results, R values indicated level of dissimilarity.

	α-diversity (P-value)	
	In vitro	*In vivo*
Faith Phylogenetic diversity	0.35	0.33
Shannon	0.57	0.23
Observed features	0.38	0.36
Evenness	0.39	0.68
Chao1	0.68	0.32

	β-diversity			
	In vitro		*In vivo*	
	R	P-value	R	P-value
Bray Curtis	0.06	0.24	0.16	0.01
Jaccard	0.12	0.86	0.12	0.03
Unweighted UniFrac	0.06	0.67	0.08	0.13
Weighted UniFrac	0.11	0.15	0.18	0.01



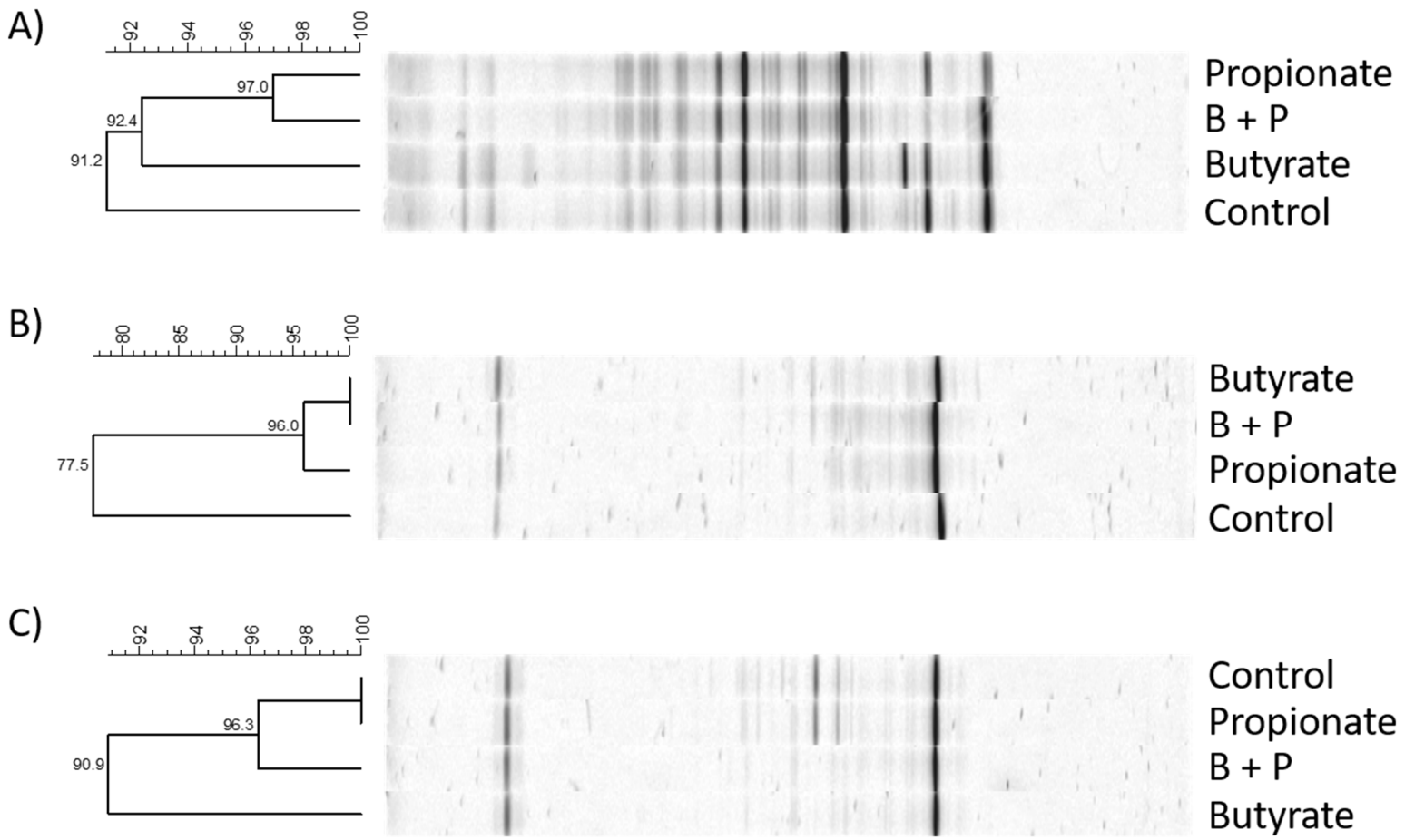
988

989 Figure 1A: Inhibitory concentration of the organic acids individually and in combination against *Aeromonas hydrophila in vitro* under two

990 different pH conditions. Upper case letters were analyzed for pH 6, and lower case letters for pH 7. Figure 1B: Inhibitory concentration of the

991 organic acids individually and in combination against *Streptococcus agalactiae in vitro* under two different pH conditions.

992



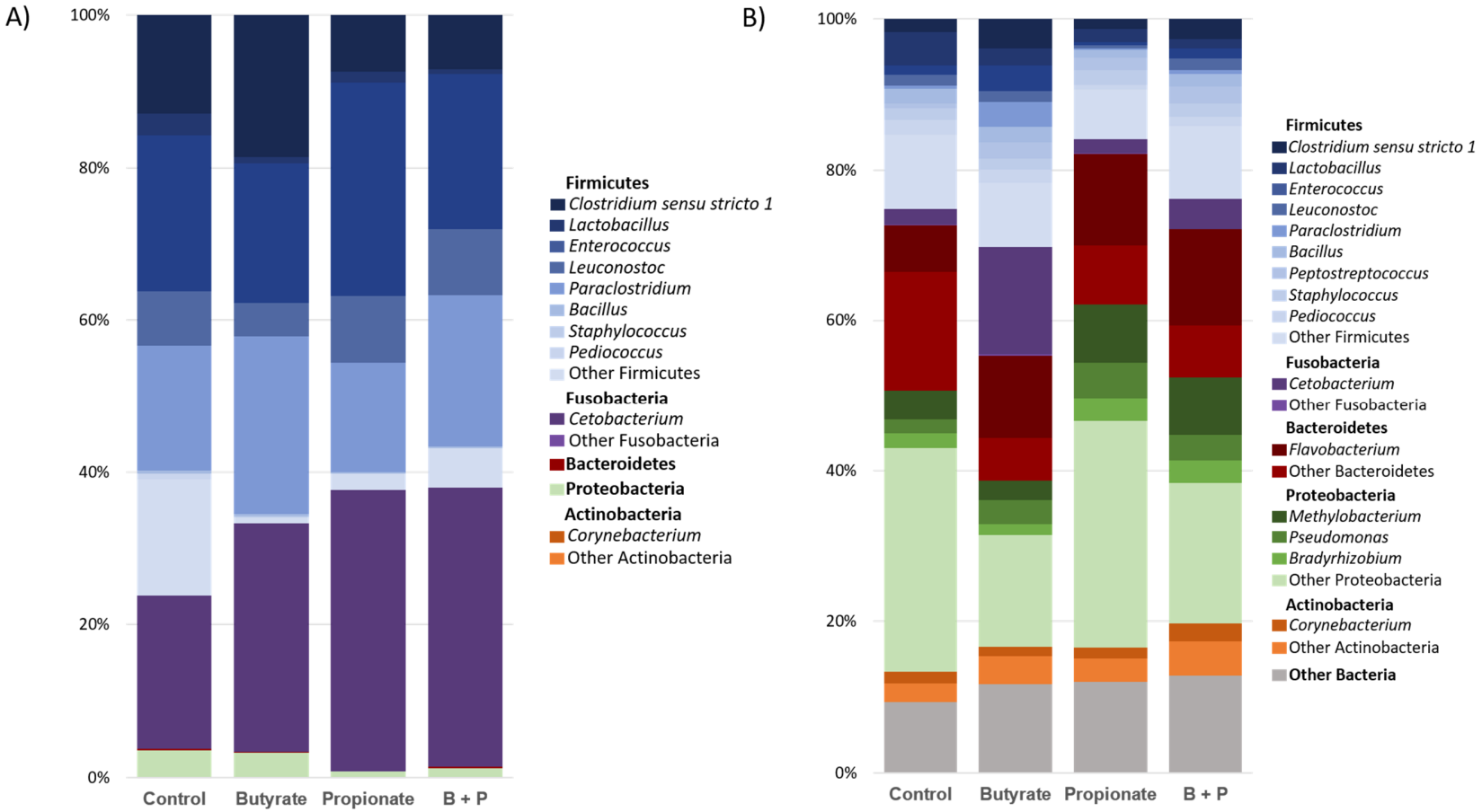
993

994 Figure 2: Denaturing gradient gel electrophoresis dendrograms of the red drum digesta microbiota. A) *In vitro* study, B) feeding trial at day 30, C)

995 feeding trial at day 60. Percentage similarity coefficient (%SC; bar) ≤ 79% = not similar populations; %SC = 80-84% somewhat similar; %SC =

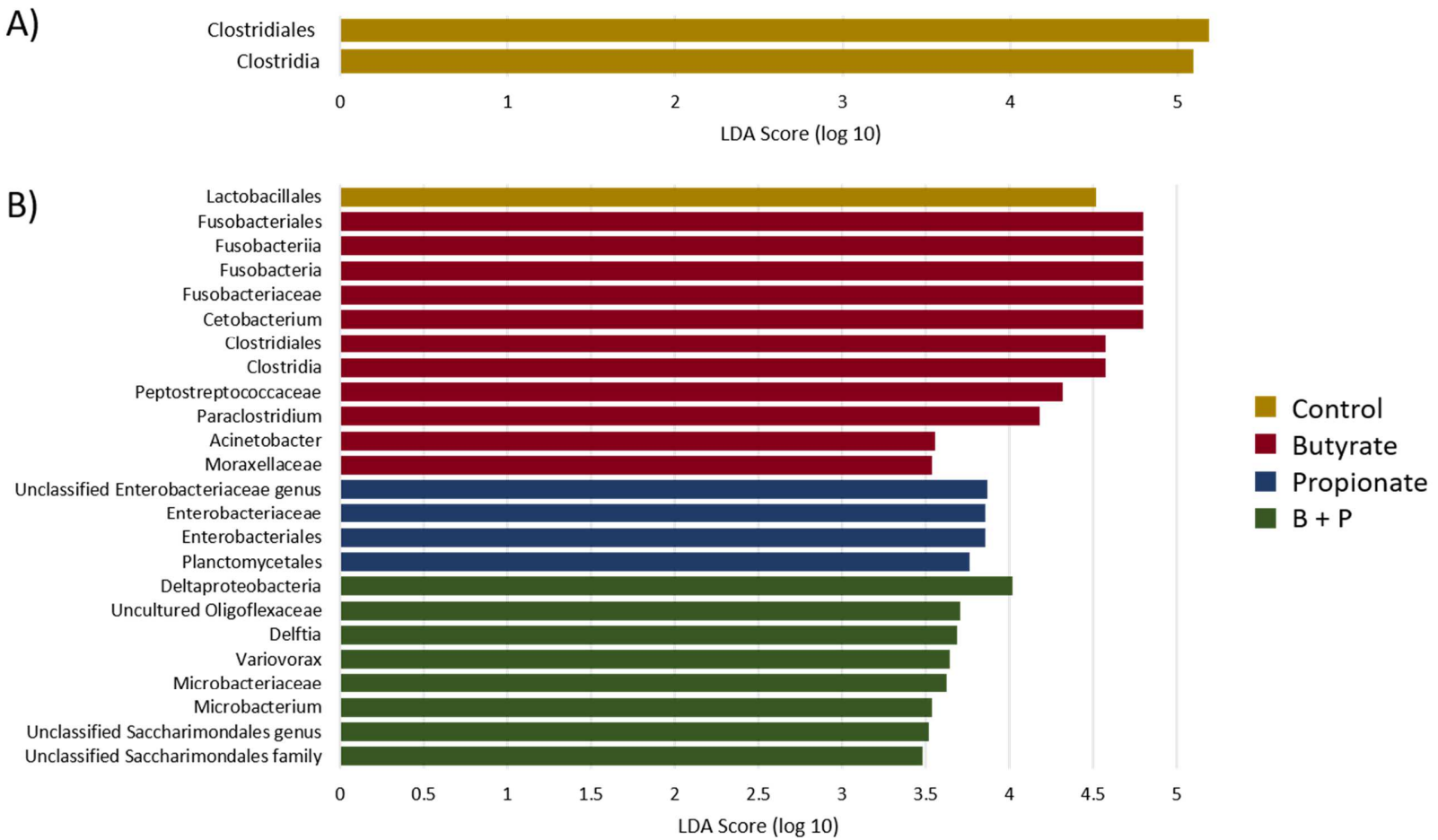
996 85-89% similar; %SC = 90-94% very similar; and %SC ≥ 95% likely the same or identical.

997



998

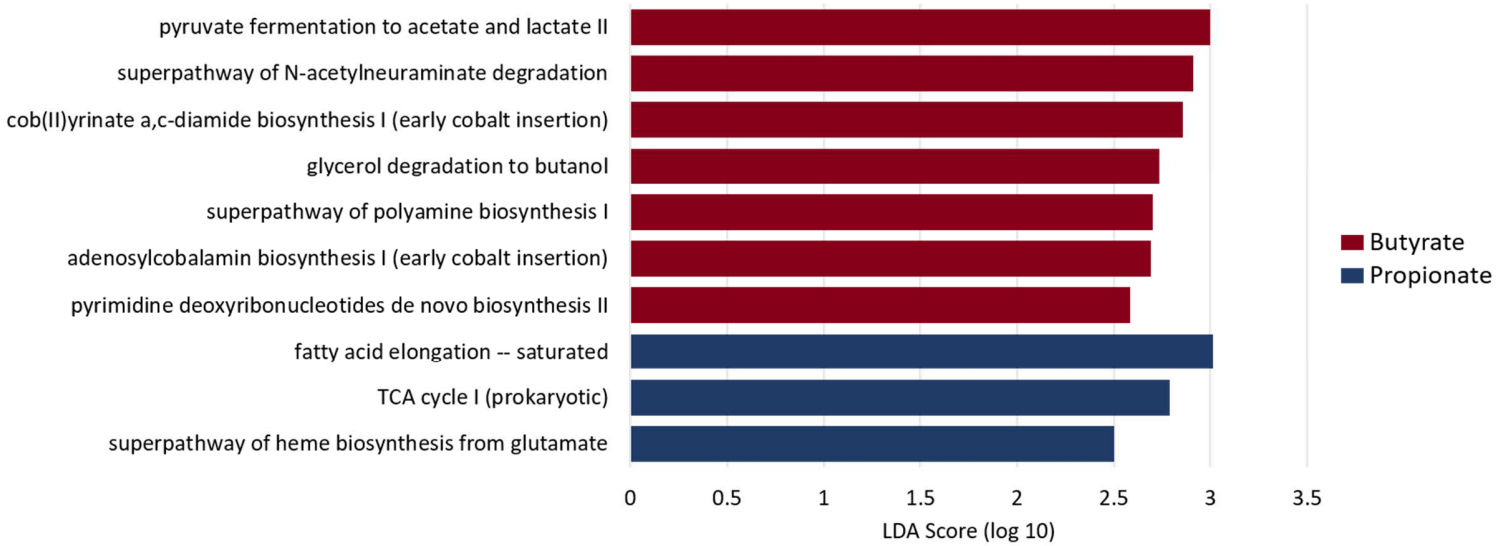
999 Figure 3: Average relative abundance of the bacterial genera from the red drum digesta in the *in vitro* (A) and *in vivo* (B) experiments.



1000

1001 Figure 4: Digesta bacteria found to be significantly differently abundant ($P < 0.05$) within treatments for the *in vitro* (A) and *in vivo* (B)

1002 experiments, determined by the linear discriminant analysis effect size (LEfSe).



1003

1004 Figure 5: Predicted functions of the digesta microbiota from the *in vivo* experiment analyzed with the linear discriminant analysis effect size

1005 (LEfSe) show significant differences ($P < 0.01$) within the dietary treatments.