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Alternative Feed Raw Materials Modulate Intestinal Microbiota and Its Relationship with Digestibility in Yellowtail Kingfish *Seriola lalandi*

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Abstract: Gut microbiota plays a crucial role in nutrient digestibility and fish health. This study aimed to investigate the effects of alternative feed raw materials on the bacterial communities in the distal intestine and its relationship with nutrient digestibility in yellowtail kingfish (YTK), *Seriola lalandi*. Two 4-week digestibility trials were conducted to evaluate fish meal (FM), two sources of poultry by-product meal (PBM-1 & PBM-2), blood meal (BLM), faba bean meal (FBM), corn gluten meal (CGM), soy protein concentrate (SPC) and wheat flour (WH). The nutrient digestibility value was determined using the stripping fecal collection method. Bacterial communities were characterized by high-throughput sequencing based on V3-V4 region of the 16S rRNA gene. The most abundant phylum identified in the present study was *Proteobacteria.* A significant change in the distal intestine was observed in fish fed diets containing CGM and BLM, characterized by a reduction of species richness and diversity. Additionally, significant correlation between nutrient digestibility and intestinal microbiota was observed. *Allivibrio*, *Vibrio*, *Curvibacter*, *Ruminococcaceae*, and *Clostridium* were positively correlated, whereas *Ralstonia* genus was negatively correlated with nutrient digestibility. This study demonstrated that intestinal microbiota could be a useful tool for evaluating the digestibility of feed raw materials; however, further culture-based study is needed to confirm this observation.

Keywords: Yellowtail kingfish; alternative raw feed materials; distal intestinal microbiota; 16S rRNA gene amplicon sequencing; digestibility; fish nutrition

1. Introduction

The increasing global demand for aquatic food products for human consumption has resulted in aquaculture being a rapidly growing industry worldwide in recent decades, supplying about 47% of aquatic food in the world [1]. The expansion of aquaculture has led to a rapid increase of aquaculture feed production. The continued high reliance on fish meal (FM) in aquaculture feed has resulted in a situation that is both ecologically and economically unsustainable. Thus, the

investigation of alternative feed raw materials has been required to satisfy the demand of the growing aquaculture sector.

Alternative feed raw materials can be for most derived from plants or land animals, and many have potential use in aquaculture diet formulation [2–6]. The most commonly used alternatives are plant raw materials (i.e., soybean products, canola, rapeseed, lupin, faba bean, corn and wheat). However, extensive use of certain plant proteins is restricted, as not only can they induce nutritional imbalances (i.e., high carbohydrate content, amino acid limitation), but they can also contain active anti-nutritional factors (ANFs) (e.g., saponins, phytic acids and protease inhibitors) that may have a negative impact on nutrient digestibility and fish health [7]. Unlike plant feedstuffs, terrestrial animal raw materials (i.e., poultry by-product meal, blood meal, meat meal and hydrolyzed feather meal) are rich in most essential amino acids [8,9]. However in some regions such as Europe, there has been limited use of these feed raw materials due to regulations and concerns regarding the introduction of transmissible spongiform encephalitis to human consumers [10].

Recently, awareness has been raised as to the crucial roles of the intestinal microbiota for nutrition and fish health, being involved in a wide range of biological processes of the host such as nutrient digestion, absorbance and immune system [11–14]. Microbial composition varies in response to environmental conditions (i.e., temperature, salinity) [15,16], development stages (i.e., larval, adult) [17] and diets (i.e., FM, FM replacement) [18,19].

Most fish microbiota studies have traditionally used culture-based techniques to characterize the wide range of microbes that colonize the fish gastrointestinal tract [20,21]. However, the traditional cultivation methods grossly underestimate the microbiome diversity as it detects only cultured bacterial species. Thus, over the past few years, several culture-independent molecular techniques such as denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) have been developed and applied to characterize microbial communities. The new powerful approach of next-generation sequencing [22] technologies allows the deep surveillance of microbial ecosystems. It has been applied to investigate the impact of diets on fish intestinal microbiota composition in several aquaculture-raised fish such as Atlantic salmon, *Salmo salar* [19,23,24], gilthead sea bream, *Sparus aurata* [25], rainbow trout, *Oncorhynchus mykiss* [9,26,27].

Yellowtail kingfish (hereafter referred to as YTK) *Seriola lalandi* is an economically important marine aquaculture species in Australia [28–32] and other parts of the world [16,33–35]. It is well suited to sea cage farming due to its rapid growth and ravenous feeding behavior. Significant gains in knowledge have been made with regard to understanding the basic nutritional requirements and nutrient digestibility of YTK [36–41] and recently several studies have investigated the microbial community of *S. lalandi* [16,17,42]; however, the impacts of the alternative feed raw materials on the intestinal microbiota and its relationship with nutrient digestibility in this species remain elusive.

The main aim of this study was to investigate the effect of alternative feed raw materials on the structure of intestinal microbiota. The secondary aim was to examine the correlation of fish gut microbiota with nutrient digestibility in YTK using high-throughput 16S rRNA gene V3-V4 hypervariable region amplicon sequencing.

2. Results

2.1. Feed Raw Materials Change the Intestinal Bacterial Community Composition

Sequencing of bacterial DNA resulted in a total of 4,681,857 paired-end 250-bp raw reads. After removing low-quality reads and chimeras, the number of quality reads was 3,208,675 reads, ranging from 23,739 to 91,414 with an average of 54,384 ± 13,417 (mean ± SD) reads per individual sample and the mean good coverage index was > 0.99 in the remaining samples, indicating the adequate depth of the sequencing (Supplementary file 3). These sequences were assigned to 4157 OTUs based on 97% similarity.

With regard to the bacterial composition, a total of nine phyla, including *Proteobacteria, Firmicutes, Actinobacteria*, *Bacteroidetes* were found among all samples in both trials. *Proteobacteria* was the most dominant phylum (45.5%–96.6%) and differed between experimental diets (Figure 1; $P <$

0.05). *Firmicutes* was the second largest phylum in all groups, comprising 1.6% to 9.4% of the assigned sequences. No significant differences in the bacterial abundance of *Firmicutes* was found among dietary groups. Families of the phylum *Proteobacteria* highly observed in YTK distal intestine were *Vibrionaceae* and *Enterobacteriaceae*. Members of family *Vibrionaceae* were the most abundant in FM (65.5%), PBM-1 (61.3%) and FBM (72.4%) in trial 1 and PBM-2 (74.2%), SPC (84.7%) and WH (90.5%) in trial 2, whereas *Enterobacteriaceae* were most dominant in fish fed the diet containing CGM (55.1%) and BLM (43.0%) (Figure 2A,B). The Venn diagram showed that cores of 75 and 48 bacterial genera were shared by the four groups of fish fed different feed raw materials in trial 1 (Figure 3A) and trial 2 (Figure 3B), respectively. Significant differences at the genus level were found among diets in both trials, highlighting *Vibrio* as highly represented in fish fed the diet containing FM (45.5%), PBM-1 (40.8%) and FBM (38.5%) in trial 1 and PBM-2 (51.3%), WH (65.3%) and SPC (63.6%) in trial 2. In contrast, fish fed diets containing CGM and BLM exhibited a significant reduction in the percentage of *Vibrio* (3.6–3.9%). Moreover, the dominance of *Ralstonia* increased when fish were fed CGM and BLM, constituting 12.6% and 38.6% respectively, compared with 1.4%–4.6% in all other groups (Figure 4A,B). We further detected the biomarkers using relative abundance of bacterial taxonomy among the experimental diets by linear discriminant analysis effect size (LEfSe). The results confirmed that family *Enterobacteriaceae*, genus *Ralstonia* were significantly more abundant in distal intestine from fish fed CGM and BLM (Supplementary file 4).

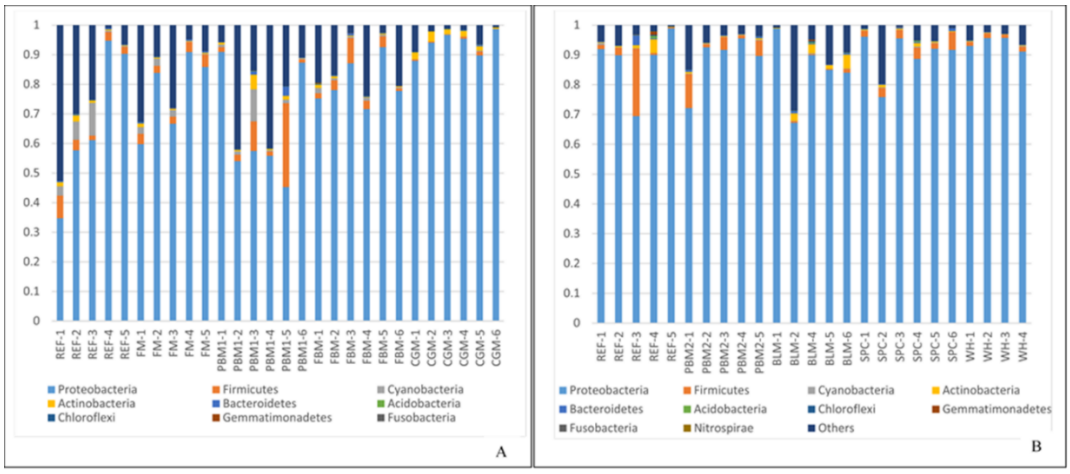


Figure 1. Relative abundance at phylum level in the intestinal microbiota of fish fed different dietary protein sources. (**A**) REF, FM, PBM1, FBM and CGM correspond to reference, fish meal, poultry by product meal, faba bean meal and corn gluten meal, respectively. (**B**) REF, PBM2, BLM2, WH, SPC correspond to REF, poultry by product meal, blood meal, soybean concentrate and wheat flour respectively.

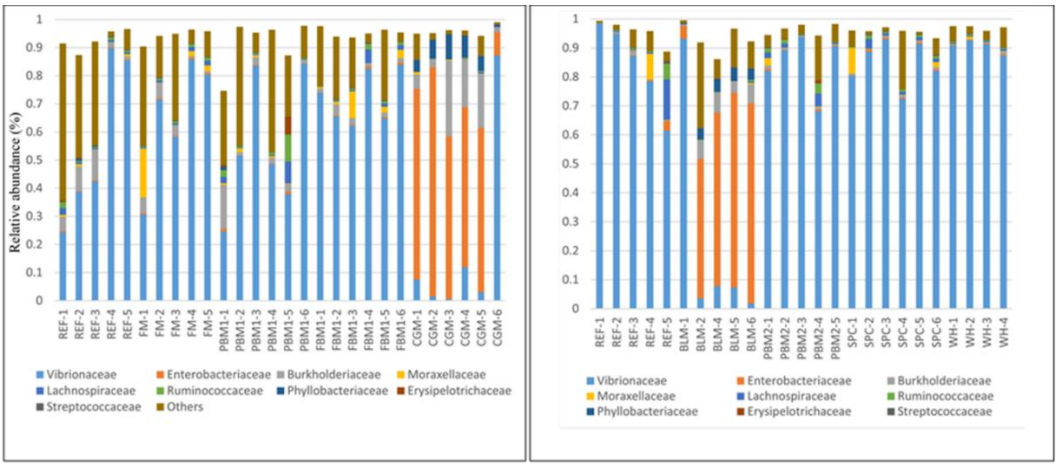


Figure 2. Relative abundance at family level in the intestinal microbiota of fish fed different dietary protein sources. (**A**) REF, FM, PBM1, FBM and CGM correspond to reference, fish meal, poultry by product meal,

faba bean meal and corn gluten meal, respectively. (**B**) REF, PBM2, BLM2, WH, SPC correspond to REF, poultry by product meal, blood meal, soybean concentrate and wheat flour respectively.

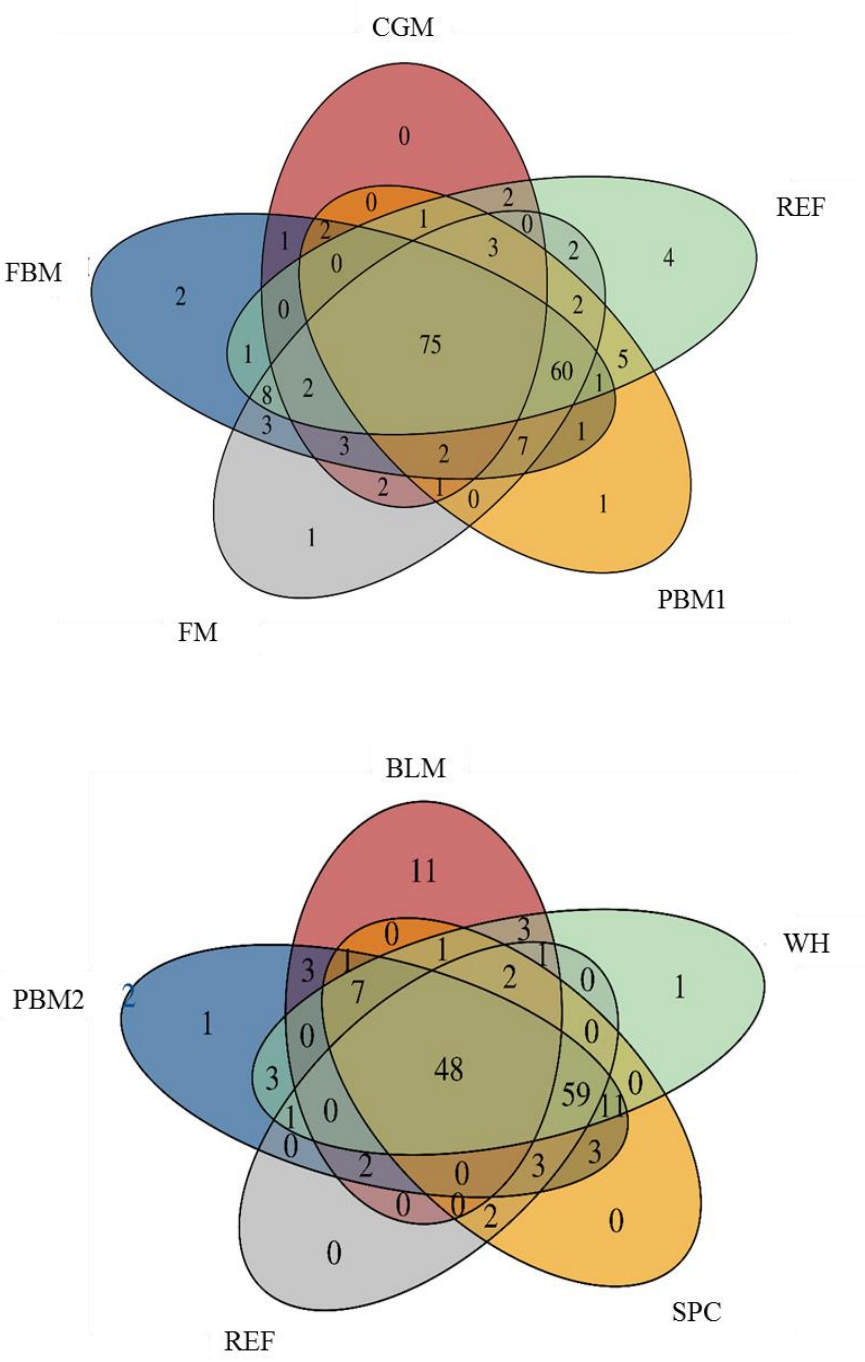


Figure 3. Venn diagram displaying the number of unique and shared OTUs in the distal intestine of YTK fed different dietary protein sources. (**A**) REF, FM, PBM1, FBM and CGM correspond to reference, fish meal, poultry by product meal, faba bean meal and corn gluten meal, respectively. (**B**) REF, PBM2, BLM2, WH, SPC correspond to reference, poultry by product meal, blood meal, soybean concentrate and wheat flour, respectively.

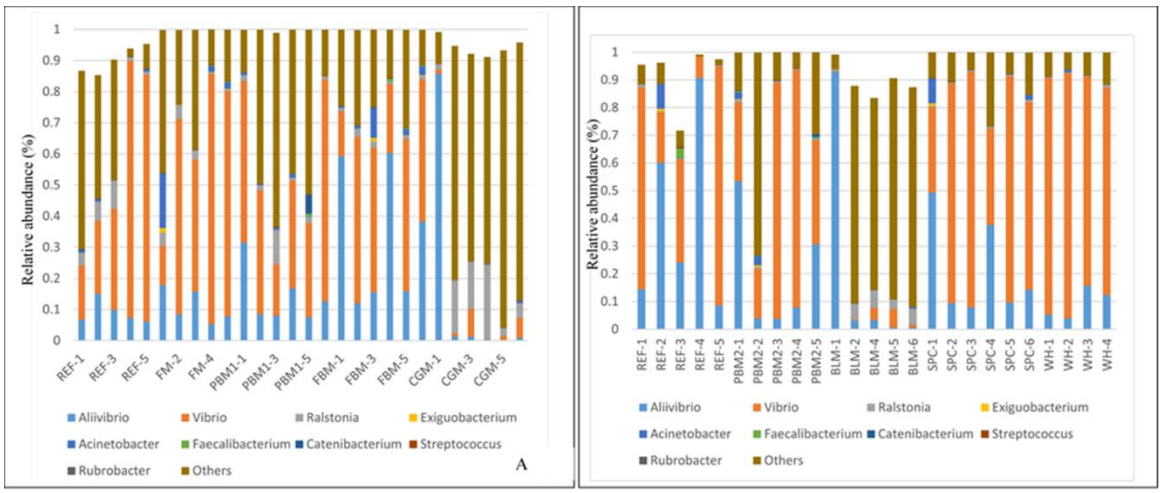
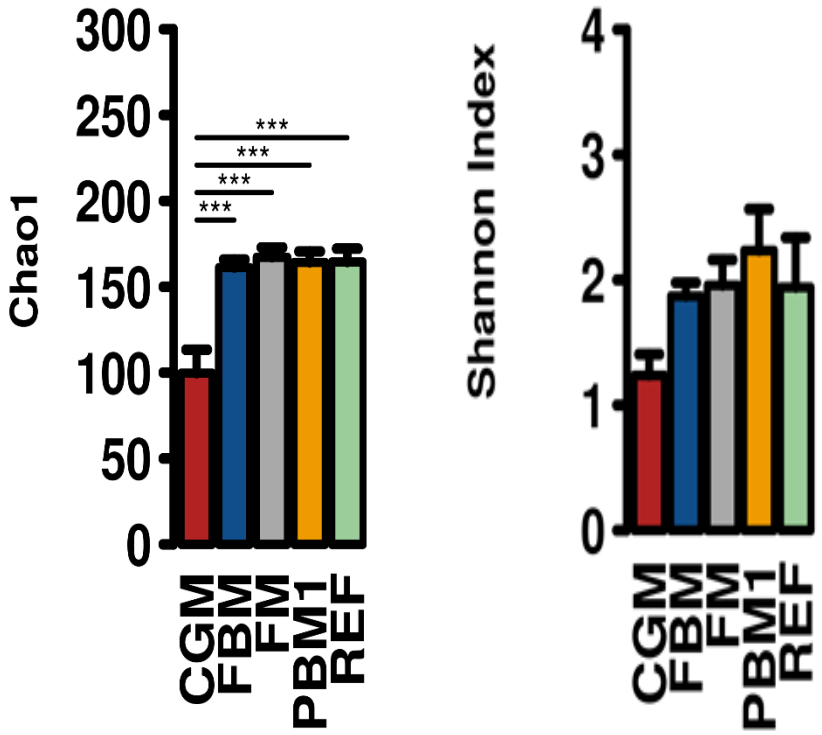
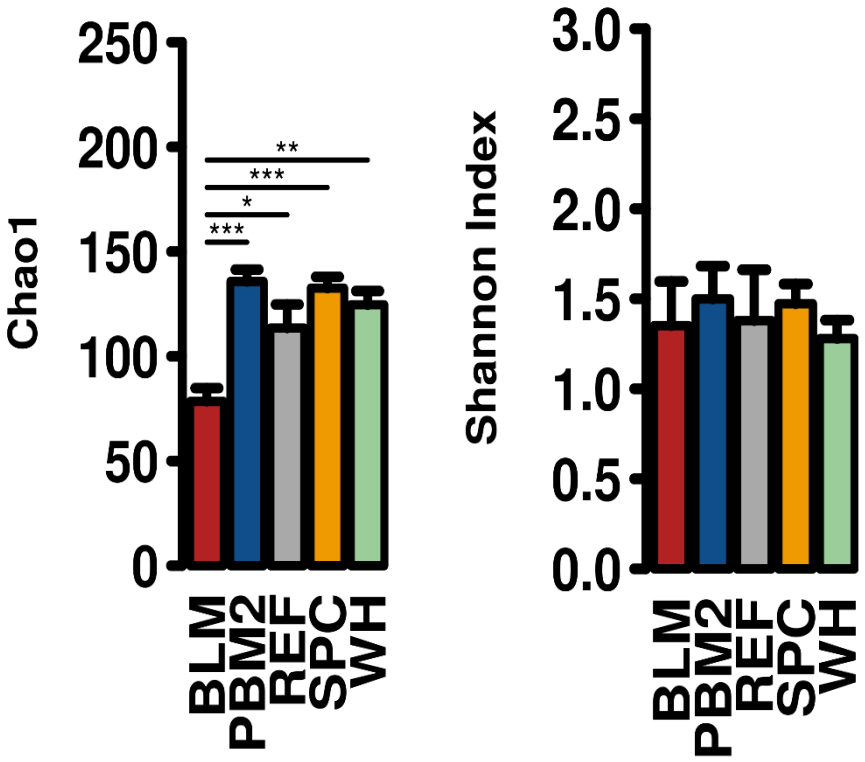


Figure 4. Relative abundance at genus level in the intestinal microbiota of fish fed different dietary protein sources. (**A**) REF, FM, PBM1, FBM and CGM correspond to reference, fish meal, poultry by product meal, faba bean meal and corn gluten meal, respectively. (**B**) REF, PBM2, BLM2, WH, SPC correspond to reference, poultry by product meal, blood meal, soybean concentrate and wheat flour, respectively.

The diversity of the distal intestinal microbiota is represented in Figures 5 and 6. Chao-1 diversity index was significantly lower in fish fed CGM and BLM compared to the other groups ($P < 0.05$ in both trial; Figure 5A,B). No significant difference in Shannon index ($P > 0.05$) was found in either trial 1 or trial 2. PCoA of Bray-Curtis distance showed a clear separation between intestinal microbiota composition of fish fed diets containing CGM and BLM compared to other groups (Figure 6A,B), as confirmed by PERMANOVA test ($R^2 = 0.492$, $P = 0.0003$ and $R^2 = 0.515$, $P = 0.0003$ in trials 1 and 2, respectively).

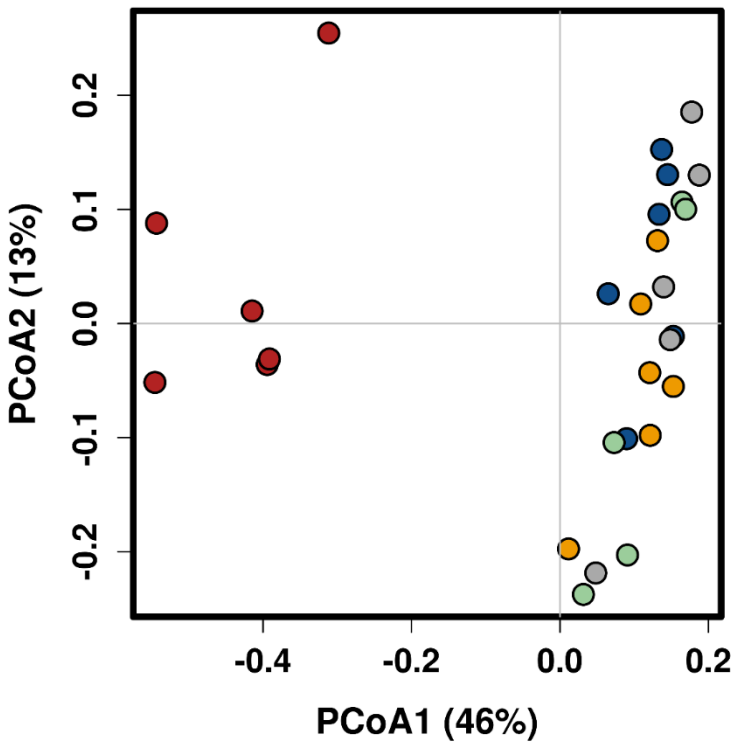


(**A**)



(**B**)

Figure 5. Alpha diversity indexes in the intestinal microbiota community of fish fed dietary protein sources. (**A**) REF, FM, PBM1, FBM and CGM correspond to reference, fish meal, poultry by product meal, faba bean meal and corn gluten meal, respectively. (**B**) REF, PBM2, BLM2, WH, SPC correspond to reference, poultry by product meal, blood meal, soybean concentrate and wheat flour respectively.



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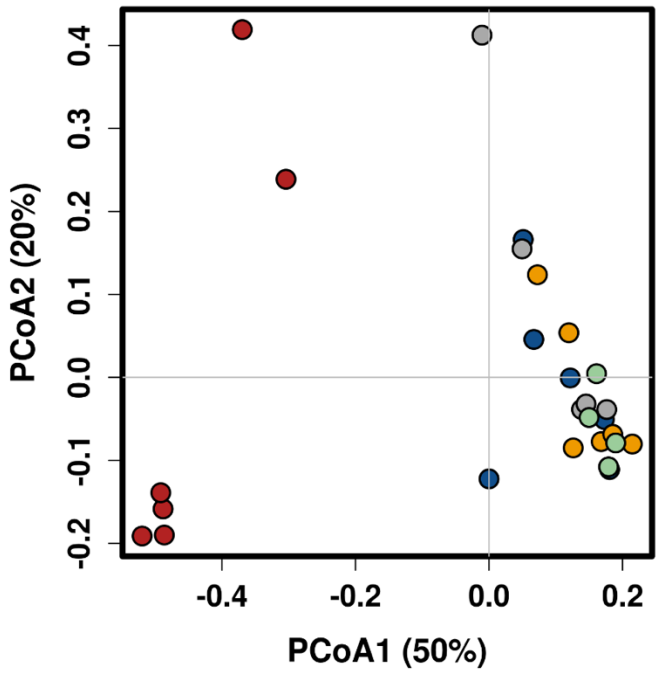

(**B**)

Figure 6. (**A**) Principal coordinates analysis of intestinal microbiota communities associated with protein sources in trial 1. REF, FM, PBM1, FBM and CGM correspond to reference, fish meal, poultry by product meal, faba bean meal and corn gluten meal, respectively; (**B**) Principal coordinates analysis of intestinal microbiota communities associated with protein sources in trial 2. REF, PBM2, BLM2, WH, SPC correspond to reference, poultry by product meal, blood meal, soybean concentrate and wheat flour, respectively.

2.2. Feed Raw Materials Modulate Predicted Intestinal Microbiome Function

Intestinal microbiome functional profiles were predicted using PICRUSt [43]. The results show that Nearest Sequenced Taxon Index (NSTI) was 0.05 ± 0.02 (mean ± SD), indicating a good relationship of the microbes in our samples to the bacterial genomes database (Supplementary file 5). A total of 327 KEGG pathways were obtained. Statistical analysis revealed that 36 KEGG pathways significantly differed in their abundance among the experimental diet groups in both trials. Differential pathway functions related to protein and amino acid metabolism, lipid metabolism, carbohydrates and energy metabolism were found. Specifically, the pathway functions related to glycine, serine, threonine, taurine cysteine and methionine metabolism, bile acid metabolism and glycosphingolipid biosynthesis, glycolysis/gluconeogenesis and TCA cycle were predicted to be more abundant in CGM and BLM fed fish compared to the others (Supplementary files 6 and 7).

2.3. Intestinal Microbiota Correlates with Apparent Nutrient Digestibility

Protein and energy digestibility of different diets deduced from our previous study [41] was used to correlate OTU abundance and digestibility. Pearson's correlation analysis in both trials showed that a total of 50 OTUs including the phylum *Proteobacteria* and 12 genera including *Alivibrio, Vibrio*, *Curvibacter*, *Ruminococcaceae*, and *Clostridium* were positively correlated with digestibility whereas *Enterobacteriaceae* family, *Ralstonia, Leifsonia* genera were negatively correlated with digestibility (Figure 7).

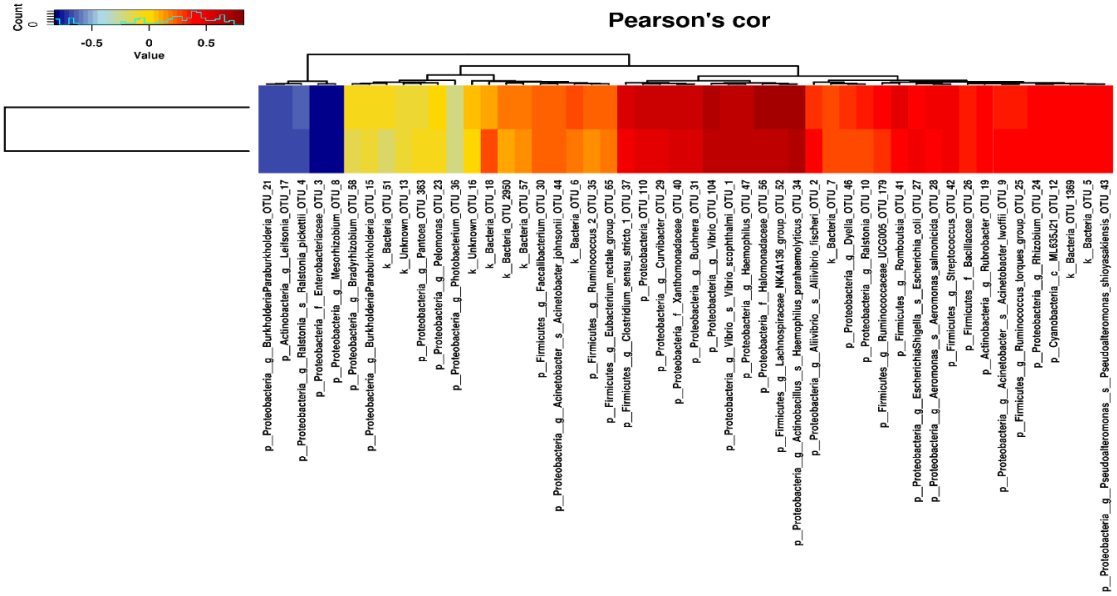

Figure 7. Correlation between OTUs and apparent protein and energy digestibility in trial 1 and trial 2. Abbreviations: ADP—Apparent digestibility protein. ADE—Apparent digestibility energy.

3. Discussion

Alternative feed raw materials are promising FM replacements in aquaculture feed formulation. As such, numerous studies have reported the effect of alternative raw materials on fish growth performance and nutrient digestibility [2,39,44-47]. Due to the microbiome being an emerging field of research, the effect of alternative feed raw materials on fish intestinal microbiota is yet to be thoroughly explored. To our knowledge, the present study is the first in-depth report on the effect of feeding alternative feed raw materials on the intestinal microbiota and its relationship with apparent nutrient digestibility in aquaculture-raised YTK using high-throughput 16S rRNA gene V3-V4 hypervariable region amplicon. This study highlighted the shift from the *Vibrionaceae* family and *Vibrio* genus in the fish fed FM, PBM, FBM, SPC and WH to the *Enterobacteriaceae* family and *Ralstonia* genus, with a reduction of species richness and bacterial diversity in fish fed CGM and BLM, which was associated with the low digestibility of these feed raw materials.

The first aim of the present study was to investigate the impact of alternative feed raw materials on the structure of bacterial community. DNA extraction was performed on distal intestine content samples, obtained from the gut after scraping the mucosa; thus, the bacterial community represented both allochthonous (transient) and autochthonous (resident). The 16S rRNA sequencing results show the most dominant phylum in the distal intestine of YTK to be *Proteobacteria*, which ranged from 45.5% to 96.6% among the experimental diet groups, this observation is in agreement with previous studies in aquaculture-raised YTK employing culture-independent techniques [48] and NGS [17]. In contrast, a previous study [16] reported that *Proteobacteria* phylum accounted for merely 20%, whereas the phylum *Firmicutes* was dominant (61%, compared with < 10% in this study) in the intestine of aquaculture-raised YTK. The differences between the two studies could be attributed to the differences in the host genetic background (Australia vs. Chile), environmental factors, the size of the experimental fish (500 g vs. 3–4 kg), the nutrient profile associated with the experimental diets, and the methodological differences (i.e., V3-V4 vs. V4 sequencing region and Nextseq vs. Ion Torrent sequencing platform). Additionally, *Proteobacteria* is also the prominent microbial phylum found in the intestine of marine fish species including Atlantic salmon, *S. salar* [49,50], gilthead seabream, *Sparus aurata* [51] and Atlantic cod, *Gadus morhua* [52]. The high presence of *Proteobacteria* in the gut microbiota of multiple fish indicates that the members of this phylum are involved in crucial host gut functions, such as digestion, absorption and against pathogen [53]. *Firmicutes* occurred in lesser percentages in our study (1.6%–9.4%), as reported in other fish gut microbiota studies [54–56]. At the family level, our results indicated that the members of *Vibrionaceae* family were highly represented (>

60%) in distal intestine of fish fed diet containing FM, PBM1, PBM2, FBM, SPC and WH, while the inclusion of CGM and BLM in the diet of our fish favored the high relative abundance of *Enterobacteriaceae*. The observed high abundance of *Vibrionaceae* could be explained by the relatively high temperature (20–22 °C) in the experimental conditions of this study. This confirmed the result from the previous studies [50,51], which reported that high abundance of *Vibrionaceae* family was seen in the intestine of Atlantic salmon, *S.salar* [50] and gilthead seabream, *S.aurata* [51]. On the other hand, the relatively high abundance of *Enterobacteriaceae*, which are involved in the metabolism of residual nitrogenous compounds that are not absorbed in foregut and midgut segments and transferred to distal intestine in the fish fed CGM and BLM; this observation could be explained by the excessive undigested protein content in the gut digesta in these groups. At the genus level, *Vibrio* was found to be the most abundant in our study (38.5%–65.3%), which is in agreement with previous studies conducted in marine fish species such as *G. morhua* [57] black rock cod, *Notothenia coriicepst* [58], grouper, *Epinephelus coioides* [59], red drum, *Sciaenops ocellatus* [15], snapper, *Lutjanusn bohar* [60] and aquaculture-raised *S. salar* [49]. *Vibrio* is a diverse genus of the phylum *Proteobacteria*, one of the most common bacterial genera in marine environment [61], this genus has a sullied reputation for well-known pathogens including *V. salmonicida, V. anguillarum, V. vulnificus, V. harveyi* and *V. parahaemolyticus*, which can cause disease in aquaculture. However, previous studies have found that many *Vibrio* species are enzyme-producing bacteria (i.e., amylase, chitinase, lipase and protease) and can act as symbionts, assisting in the breakdown of dietary components [62–65]. Additionally, several species including *V. alginolyticus* and *Vibrio sp.* Strain NM10 have been shown to work well as probiotics (health-promoting) for Atlantic salmon, *S. salar* [66,67]. In the present study, no inflammation (disease) signs were observed in the fish fed test diets (data not shown) during experimental period. The high abundance of *Vibrio* genus among experimental diets (except CGM and BLM) in this study might be attributed by the beneficial roles in producing hydrolytic enzymes to breakdown the dietary components. This result suggests further investigation on the enzyme-producing gut bacteria isolated from YTK. Interestingly, in the present study, we found the dominance of the *Ralstonia* genus, where most sequences of this genus were closely related to sequences identified as *Ralstonia picketti* (formerly known as *Burkhoderia picketti* or *Pseudomonas picketti*) in diet containing CGM and BLM. The members of *Ralstonia* genus have been found in the gut content of cultured yellow catfish, *Pelteobagrus fulvidraco* [68], rainbow trout Oncorhynchus mykiss [69], European seabass, *Dicentrarchus labrax* [70]. A previous study reported that *Ralstonia* species are opportunistic pathogens in Nile tilapia, *Oreochromis niloticus*, African catfish, *Calarias gariepinus* [71]. However, the specific roles of this bacterial group in fish remain unclear. Further culture-based studies on the role of *Ralstonia* will be required to confirm this observation.

With regard to bacterial diversity, we found that replacing FM with PBM, FBM and WH did not induce significant changes in gut microbial diversity. This is in agreement with previous studies on Asian seabass, *L. calcarifer* [72], gilthead seabream, *S. aurata* [73]. However, the Chao1 species richness was significantly reduced in fish fed CGM and BLM in present study. It is important to note that high bacterial diversity is considered to have positive effect on gut health, since species-rich communities are thought to compete pathogens for nutrient and prevent the colonization by pathogens [27,74,75]. Several mechanisms are proposed to explain the observed low species richness in CGM and BLM. First, the diet change could have induced the differences in the intestinal environment and thus reduce bacterial species in the digestive tract [76]. Secondly, the excessive of indigestive protein content of diet might reduce the microbial diversity. Thirdly, the high inclusion (30%) of CGM in the diet could negatively influence the diversity of bacterial groups, which is associated with the activities of brush-border digestive enzymes [77]. However, further research is needed on the link between dietary component and bacterial diversity.

The distribution of predicted functional capabilities among bacterial communities was generated in PICRUSt [43]. However, the reliability of such tools should be well validated. The NSTI scores were widely used to evaluate the accuracy of the metagenomics prediction. A previous study on YTK [16] showed the same NSTI as the present study (0.05 ± 0.02), which was less accurate compared to human intestine metagenomes prediction (NSTI < 0.03) [43], possibly explained by the

fact that some of the functional capacities of YTK bacterial communities are unknown. An interesting finding in our study was the high relative abundance of predicted bacterial functions related to amino acids metabolism, bile acids metabolism and glycosphingolipid biosynthesis, glycolysis/gluconeogenesis and TCA cycle were predicted to be more abundance in CGM and BLM fed fish compared to the others. One possible interpretation of our results is that the host intestine environment of fish fed those dietary groups favors the proliferation of intestinal bacteria that are more efficient in metabolizing amino acids and carbohydrates. This finding is in agreement with [50], who stated that feeding a carbohydrate rich diet to *S.salar* promotes intestinal bacteria capable of metabolizing carbohydrates as a major energy source.

The second goal of this study was to preliminarily correlate the changes in distal intestine microbiota with nutrient digestibility. Our study preluding this experiment [41] showed that inclusion of PBM, FBM and WH had no negative effect on digestibility; however, CGM and BLM had poor nutrient digestibility, and interestingly, in present study, it stands in a clear correlation with the strikingly altered distal intestinal microbial community in these diets, characterized by a significant higher abundance of *Enterobacteriacea*e family and *Ralstonia* genus in low digestibility diets. To our knowledge, the present study is the first to investigate the relationship between microbiota community and apparent nutrient digestibility in aquaculture species. In our experimental conditions, we found that correlation between microbiota and apparent nutrient digestibility (i.e., protein, energy) were significant. We found 50 out of 4157 OTUs were significantly correlated with apparent protein and energy digestibility with a highest absolute correlation value reaching 0.82. These correlation values were generally higher than previous studies in human, sows and growing pigs [78–80]. This confirms that the fish intestinal bacterial community plays important roles in fish nutrient digestion. Specifically, phylum *Proteobacteria* and 12 genera including *Vibrio* and *Clostridium* were positively correlated with protein and energy digestibility, potentially due to their capacity to produce digestive enzymes that metabolize the dietary nutrients more efficiently. For example, many *Vibrio* species are hydrolytic-enzyme producing, which can act as symbiont assisting in the breakdown of dietary components. This observation was reported in many previous studies [76]. *Clostridium* is a very common genus belonging to phylum *Firmicutes*, shown to contribute to host's nutrition by supplying fatty acids and vitamins. Previous studies reported that *Clostridium* sp. displays enzyme activities of acids and alkaline phosphatases, C4 and C8 esterases, C14 lipases and glycoses in southern flounder, *Paralichthys lethostigma* [81]. The negative correlation between *Enterobacteriaceae* and *Ralstonia* with protein and energy digestibility could be partly due to the indirect effect of the reduction in microbial species richness, which led to colonize of some opportunistic pathogenic strains resulted in the decrease of nutrient digestibility. However, the role of specific bacteria in digestive function by culture-based technique is needed.

In conclusion, our study contributes to the growing body of work on aquatic animal microbiomes, especially as this is the first study to systematically investigate the effect of feed raw materials on intestinal microbiota and its relationship with apparent nutrient digestibility. We found that *Proteobacteria* was the most dominant phylum in distal intestine of YTK. The significant change in the microbiome occurred in fish fed diets containing CGM and BLM, characterized by the most relative abundance of genus *Ralstonia*, a reduction of Chao-1 diversity index and a clear differentiation in the intestinal microbial populations (PCoA, PERMANOVA, $P < 0.05$), which associated with low apparent nutrient digestibility of these feed raw materials. Additionally, we found *Proteobacteria* and 12 genera including *Allivibrio, Vibrio, Curvibacter, Ruminococcaceae* and *Clostridium* positively correlated whereas *Enterobacteriaceae* family and *Ralstonia* genus negatively correlated with protein and energy digestibility. Taken together, these findings suggest that the inclusion of CGM and BLM had negatively affected the intestinal microbial community which could be one of the reasons for the reduced digestibility of those feed raw materials in YTK. We also demonstrated here that there is a relationship between intestinal microbiota and apparent nutrient digestibility, which suggests that intestinal microbiota could be useful strategy for evaluating the digestibility of feed raw materials; however, further culture-based study is needed to confirm this observation.

4. Materials and Methods

4.1. Ethics Statement

All experiments involving fish were approved by the Animal Ethics Committee of New South Wales, Department of Primary Industry (DPI) Fisheries Animal Care (Aquaculture Nutrition ACEC Authority 93/5- Port Stephens) and the University of the Sunshine Coast (AN/S/16/46).

4.2. Experiment Design

The present study is a part of a larger study evaluating the effects of alternative feed raw materials on the digestibility, gut microbiota and transcriptomics response. Two 4-week digestibility trials were conducted as described previously [41]. In this study, eight ingredients were selected for testing the impacts on intestinal microbiota based on their popularity in Australia's aquafeed industry and nutrient digestibility values obtained from previous study [43]. Trial 1 investigated the intestinal microbiota of fish fed by prime fish meal (FM), poultry by-product meal 1 (PBM-1), faba bean meal (FBM) and corn gluten meal (CGM). Trial 2 determined the gut microbiota of fish fed by poultry by-product meal 2 (PBM-2), blood meal (BLM), soy protein concentrate (SPC) and wheat flour (WH). The same reference diet based on FM was used in both experiments. PBM-2 had higher content of protein, lipid and most amino acids, whereas it had lower content of ash than PBM-1. The composition and digestibility value of feed raw materials are presented in Supplementary file 1. Diet formulation, and proximate and amino acid compositions of experimental diets are reported in Supplementary file 2. Prior to the trials, YTK were reared in large holding tanks (10 kL) and fed a commercial marine finfish diet (Ridley Aquafeed Pty. Ltd., Narangba, QLD, Australia). Six sub-adult YTK weighing 573.9 ± 17.6g in trial 1 and 513.7 ± 17.1 in trial 2 (mean ± S.D), respectively, were placed into replicate 200 L cages at the beginning of each trial. Cages (n = 3) were randomly assigned to each of the experimental diets examined in each trial. The average water temperature and dissolved oxygen concentration of both trials were 20.3 ± 0.1 °C and 9.33 ± 0.3 mg L-1, respectively, the salinity ranged from 36 to 37ppt and the photoperiod was 14 L: 10D.

4.3. Sampling

At the termination of each trial, fish were euthanized with AQUI- S (50–60 mg L-1 AQUI-S, New Zealand.). Six fish per treatment were sampled and swabbed with a 70% ethanol solution (Chem Supply, Australia) around the ventral region to disinfect the area prior to dissection. The abdominal cavity was then opened at the ventral line and the intestine (excluding pyloric caeca) was aseptically removed and opened longitudinally. The intestinal bacteria were then collected by scraping intestinal mucosa with sterile cotton swab and then placed in 1.5 mL tube of RNAlater (Ambion, Thermo Fisher Scientific, Australia) and stored at 4 °C overnight before being transferred to −80 °C until being analyzed for microbial diversity profiling.

4.4. DNA Extraction and High-Throughput Sequencing of Bacterial 16S rRNA Genes

Genomic DNA was isolated from distal intestinal content samples using a Fast DNA Spin Kit (MP Biomedical, USA) according to the manufacturer's instructions with the following modifications: 100 mg of the samples was added in 2 mL Lysing Matrix E tube containing 825 μL Sodium Phosphate buffer and 275 of PLS solution. Samples were then homogenized in Fast Prep-24 (MP Biomedical, USA) at setting 6.0m/s for 40 s and then centrifugation time was extended for 15 min. The incubation time after adding 250 μL of PPS solution increased from 10 to 30 min. DNA concentration was measured by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). DNA degradation and potential contamination were tested using 1% Agarose Gel Electrophoresis at 100 voltage (V) for 40 min. Extracted DNA was stored at −20 °C for downstream analysis.

The hypervariable V3-V4 region of the 16S rRNA gene was amplified using bacterial primer 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′) with a specific barcode for each sample. Each PCR reaction was carried out in 25 μL using 2 μL of DNA

template (concentration was ranged from 3.82 ng/μL to 30 ng/μL), 5 μL of Phusion® High-Fidelity PCR master mix (New England Biolabs) and 1 μL each of forward and reverse primers (10 μM). A negative control without DNA template was included in the run. The cycling procedure was run as follows: initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation for 30 s at 95 °C, annealing at 53 °C for 30 s and an extension at 72 °C for 30 s and then followed by a final extension at 72 °C for 10 min.

PCR products were examined using 1% Agarose Gel Electrophoresis at 100 V for 40 min for detection. Samples with a bright main strip between 400–450 bp were chosen for the downstream analysis. PCR products were mixed in equi-density ratios and then purified using Qiagen Gel Extraction Kit (Qiagen, Germany). Libraries were then generated with NEBNext® UltraTM DNA Library Prep Kit for Illumina following manufacturer's recommendation and index codes were added. The library was quantified via Qubit2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyser 2100. Finally, the library was sequenced on an Illumina Hiseq2000 platform and 250 bp paired-end reads were generated at Novogene Genome Sequencing Company (Hong Kong, China).

4.5. Sequencing Data Processing

Paired-end reads were assigned to samples based on their unique barcode and truncated by trimming off the barcode and primer sequence. Paired-end reads were merged using Fast Length Adjustment of Short read (FLASH v1.2.7) which was designed to merge paired-end reads when at least some of the reads overlapped the read generated from the opposite end of the same DNA fragment, the splicing sequences were then called raw reads. Quality filtering on the raw reads was performed under specific filtering conditions to obtain the high-quality clean reads according to Quantitative Insights into Microbial Ecology (QIIME v1.7.0) quality control process. The reads were compared with the Gold database using UCHIME algorithm to detect chimera sequences, and then the chimera sequences were removed and filtered reads were finally obtained. The 16S rRNA genes sequences were deposited in NCBI Sequence Read Archive (SRA) from SRX7796910 to SRX7796968 under BioProject PRJNA608540.

4.6. Operational Taxonomic Units (OTUs) Cluster and Species Annotation

Filtered reads were then performed by Uparse (v7.0.1001). Sequences with ≥ 97% similarity were assigned to the same OTUs. The GreenGene database (v13.5) was used based on RDP classifier (2.2) algorithm to annotate taxonomically. OTUs assigned to phylum Cyanobacteria were excluded from the final dataset as they were considered as plant contaminant.

To analyze the data, the 97% OTU table were rarefied to 10,000 sequence reads per sample and rare taxa with less than 0.01% relative abundance were removed before statistical analysis. The α-diversity indices (i.e., Chao1 and Shannon) were calculated and a one-way ANOVA followed by pairwise comparisons using Tukey's test was used to examine significance between groups. Chao 1 is a measure of total richness. Shannon index reflects species number and evenness. For β-diversity analysis, the rarefied OTU table was square root transformed before measuring community similarity, calculated as non-phylogenetic Bray-Curtis distances matrix and visualized through principal coordinates analysis (PCoA). The significance of the impact of feed raw materials on the overall bacterial communities was examined by the permutational analysis of variance (PERMANOVA) statistical test. ANOVA was also used to test for the differences in the abundance of individual taxa among experimental diets. Pearson's correlation tests were used to analyze correlations between individual OTUs and apparent nutrient (i.e., protein and energy) digestibility, which was presented in our previous study [41]. All tests were corrected for multiple comparisons using false discovery rate (FDR) correction by Benjamin and Hochberg. A Venn diagram for shared microbiomes was generated. Linear discriminant analysis effect size (LEfSe) was used to detect the biomarkers (LDA >3) in relative abundance of bacterial taxonomy. All statistical analyses were performed in Calypso (v.8.84, http://cgenome.net:8080/calypso-8.84) with total sum normalization (TSS) for statistical analysis and Cumulative Sum Scaling normalization (CSS) for multivariate test. P-value < 0.05 were regarded as statistically significant.

4.7. Predicting Function and Metagenome Contribution

The inferred metagenomes were predicted using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) (v1.1.3) [43]. Closed reference picked OTU table against Greengene v13_5 database was used as input for PICRUSt following the workflows recommended by developers, including normalization by dividing each OTU by the known or predicted 16S rRNA copy numbers and then predicted metagenomes were grouped into categories by function. Predicted functional pathways were annotated using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The metagenomes predictions accuracy was assessed by computing the Nearest Sequenced Taxon Index (NSTI), which is an index that indicates the relationship of the microbes in a particular sample to the bacterial genomes in a database. Determination of the significant differences in the predicted function was performed in STAMP (v.2.1.3).

Supplementary Materials: The following are available online at www.mdpi.com/2410-3888/5/2/14/s1, Supplementary file 1: The nutritional composition ($g \cdot kg^{-1}$ or $MJ \cdot kg^{-1}$ dry matter basis) and nutrient digestibility value (%) of feed raw materials. Supplementary file 2: The formulation and nutrient composition of experimental diets. Supplementary file 3: Raw sequence reads of individual sample. Supplementary file 4: NSTI of individual sample. Supplementary file 5: Linear discriminant analysis effect size (LEfSe) differences between OTU abundances in trial 1 and trial 2. Supplementary file 6: significant functional pathways of microbial community in trial 1. Supplementary file 7: significant functional pathways of microbial community in trial 2

Author Contributions: C.T.M.D. performed the experiment, DNA extraction, data collection, data analysis interpretation, wrote the initial version of the manuscript, M.S. and R.S. provided feed formulation and ingredients for the trial, M.B., I.P., M.S., T.V., A.E. were responsible for designing the experiment and contributed to the drafting of manuscript. All authors have read and agreed to the published version of the manuscript.

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