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Betiku, Omolola C., Carl J. Yeoman, T. Gibson Gaylord, Glenn C. Duff, Timothy Hamerly, Brian Bothner, Stephanie S. Block, and Wendy M. Sealey. "Differences in amino acid catabolism by gut microbes with/without prebiotics inclusion in GDDY-based diet affect feed utilization in rainbow trout." Aquaculture (September 2017). DOI: 10.1016/j.aquaculture.2017.09.006.

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PII: S0044-8486(17)31230-9
DOI: doi: 10.1016/j.aquaculture.2017.09.006
Reference: AQUA 632808

To appear in: *aquaculture*

Received date: 15 June 2017
Revised date: 5 September 2017
Accepted date: 6 September 2017

Please cite this article as: Omolola C. Betiku, Carl J. Yeoman, T. Gibson Gaylord, Glenn C. Duff, Timothy Hamerly, Brian Bothner, Stephanie S. Block, Wendy M. Sealey , Differences in amino acid catabolism by gut microbes with/without prebiotics inclusion in GDDY-based diet affect feed utilization in rainbow trout, *aquaculture* (2017), doi: 10.1016/j.aquaculture.2017.09.006

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Differences in Amino Acid Catabolism by Gut Microbes with/without Prebiotics Inclusion in GDDY-based Diet Affect Feed Utilization in Rainbow Trout

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Abstract

There is the need to enhance feed efficiency and growth of rainbow trout to reduce production costs of cultured fish. This study conducted a 3 x 4 factorial experiment with three graded levels of grain distiller dried yeast (GDDY) protein (0%, 50%, 75%) as replacement for fishmeal and four different prebiotics inclusion levels (0% (control), 0.4%, 1% mannooligosaccharides (MOS), and 1% GroBiotic A). The feeding trial was conducted for 12 weeks during which fish were fed daily to apparent satiation. Growth of rainbow trout was not affected by replacement of fishmeal with GDDY, but feed conversion ratio ($P<0.0001$) was greater at the highest level of GDDY inclusion. Increasing GDDY inclusion significantly increased feed intake ($P<0.00015$), which resulted in poor feed utilization. Acetic ($P=0.1994$), propionic ($P=0.8037$), butyric ($P=0.6268$), valeric ($P=0.5877$), and isovaleric ($P=0.5919$) acids profiles did not differ by diet nor with inclusion of MOS or GroBiotic A. Whole shotgun metagenomic analyses of the gastrointestinal tract (GIT) microbiota revealed enrichment in the fungal phyla *Ascomycota* and *Basidiomycota* and the bacterial phylum *Actinobacteria* in the GDDY-fed fish compared to those fed the control fishmeal-based diet, which may be reflective of the species endogenous in GDDY. Microbial genes involved in branched-chain amino acid metabolism (glutamate, glutamine, aspartate) ($P=0.028$) and glutamate dehydrogenase clusters ($P=0.0192$), were also elevated in the fish fed the 75% GDDY-based diet. The results from this study indicate the potential for microbially-mediated catabolism of the non-essential amino acids, and suggest this activity may significantly influence efficient utilization of dietary nitrogen in the yeast-based protein diet.

Keywords: Prebiotics, grain distiller dried yeast, alternative protein, metagenomics, amino acids, rainbow trout

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1. Introduction

Prebiotics, which are feed ingredients that are not endogenously digestible by the host but selectively stimulate the growth of beneficial microbes in the host, have been reported to improve feed efficiency, growth rate, and weight gain in rainbow trout (Bagheri et al., 2008; Merrifield et al., 2010). Human studies have shown prebiotics can improve the production of short chain fatty acids thereby enhancing the supply of energy (Ríos-Covián et al., 2016). Positive effects of mannoligosaccharides (MOS) from *Saccharomyces cerevisiae* in fish feeding studies include improved growth, survival and immune responses (Sealey et al., 2007; Ringø et al., 2010; Mansour et al., 2012). However, reports of these benefits are inconsistent (Genc et al., 2007; Welker et al., 2007; Grisdale-Helland et al., 2008; Sado et al., 2008; Torrecillas et al., 2011; Sealey et al., 2015a). Grobiotic®-A (GroA), a mixture of partially autolyzed brewer's yeast, dairy ingredient components and dried fermentation products, is another commercially available prebiotic that is gaining attention in fish nutrition studies (Li and Gatlin, 2004; Burr et al., 2009; Sealey et al., 2015a). Although GroA is reported to lower pathogenic bacteria, information on how it specifically alters microbial compositions in the gastrointestinal tract (GIT) in these fishes is lacking (Li and Gatlin, 2004; Burr et al., 2009).

The GIT, especially, the small intestine, is the active site for digestion and metabolism of endogenous and exogenous amino acids in fish and other animals (Adibi and Mercer, 1973; Ash, 1980; Dabrowski and Dabrowska, 1981; Attaix and Arnal, 1987; Betiku et al., 2017). The mucosa of the small intestine catabolizes non-essential amino acids (glutamine, glutamate and aspartate), which supplies oxidative fuels to the GIT mucosa and also serve as precursors for intestinal synthesis of proteins, pyrimidine, purines, proline, nitric oxide and glutathione (Reeds et al., 2000). In addition to catabolism of these non-essential amino acids, essential amino acids, including the branched-chain amino acids (BCAA),

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threonine, lysine and phenylalanine are also oxidized (Adibi and Mercer, 1973; Wu, 1998); and the rate of metabolism of these amino acids in the intestine is determined by enteral and parenteral amino acids availability in the arterial blood (Burrin, 2002). Fermentation of the BCAA: valine, leucine and isoleucine into short chain fatty acids (SCFA) for use by the GIT microbiota also has been observed (Ríos-Covián et al., 2016).

Although it is not fully known in fish how dietary protein sources affect the catabolism of amino acids and their subsequent availability for use by the GIT mucosa, this information may be of great importance in achieving improved nitrogen utilization when alternative protein ingredients are used in aquaculture (Davila et al., 2013). In addition, amino acid catabolism by the GIT mucosa plays an important role in modulating absorbed amino acids entry into the portal circulation, with differences in the appearance of amino acids in the portal circulation that may not reflect their availability to the extra-intestinal tissue. In essence, amino acid catabolism plays a significant role in the status of protein turnover. Specifically in the model aquaculture fish species rainbow trout, postprandial amino acids concentrations are influenced by protein sources, resulting in differences in appearance between essential amino acids and non-essential amino acids (Walton and Wilson, 1986; Larsen et al., 2012).

Recent evidence by Chan (2016) suggests that hepatic glutamate dehydrogenase (GDH) is the endogenous catabolism enzyme responsible for poor nitrogen retention efficiency observed in adult trout having higher concentration of this enzyme compared to the larval fish. Differences in concentration of GDH may be associated with increased energy demand in the adult fish through increasing hormonal regulation of the digestive enzymes (Rønnestad et al., 2013). GDH is found in most living organisms including microbes, where it catabolizes a reversible transdeamination reaction of glutamate to α-ketoglutarate (Reeds et al., 2000). GDH has been detected in rainbow trout and its activity is negatively correlated

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with nitrogen retention efficiencies (Davies et al., 1997; Mambrini et al., 1999; Martin et al., 2003). However, the mechanistic process by which catabolized amino acids are deaminated is not fully understood, especially when there are exchange of amino acids between the GIT microbiota and the host. Since nitrogen retention involves a complex interaction between protein synthesis and protein degradation, shotgun sequencing was employed to analyze microbial diversity and gene functions associated with amino acids synthesis and catabolism within rainbow trout small intestine fed Grain distiller dried yeast (GDDY).

GDDY is a single-cell yeast-based protein ingredient with high protein content and has been successfully used as a protein source in rainbow trout nutrition, partially replacing fishmeal when supplemented with synthetic amino acids (Hauptman et al., 2014a; Hauptman et al., 2014b; Sealey et al., 2015b). However, inclusion levels above 15 - 18% have had significant impacts on efficient feed utilization in rainbow trout suggestive of a potential nutritional stressor associated with this protein source (Hauptman et al., 2014a; Hauptman et al., 2014b; Sealey et al., 2015b). In rainbow trout, the influence of prebiotics on activity of the GIT microbial composition is yet to be determined; however, beneficial effects of prebiotics have been most often observed in nutritionally stressing diets. Therefore, the present study employed a metagenomic technique to investigate nutrient utilization in the midgut of rainbow trout. In particular, microbial contributions to amino acid catabolic processes were assessed in trout fed a nutritionally stressing diet with two common prebiotics in order to provide a more complete understanding of the mechanistic processes involved in amino acid utilization when alternative proteins are employed in aquaculture.

2. Materials and methods

2.1. Experimental design

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A 3 x 4 factorial design experiment was conducted with twelve experiments diets (Table 1) to contain three increasing levels of GDDY (0, 50%, 75%) as a replacement of fishmeal and four prebiotic treatments (0%, 0.4% MOS, 1% MOS, 1% GroA). All diets were labelled using yttrium as inert marker and were formulated at 40% crude protein and 20% crude lipid, supplemented with lysine, methionine and threonine balanced to the rainbow trout fillet ideal amino acid targets of 3.8 %, 1.3 % and 2.1%, respectively, described by Gaylord and Barrows (2009). All fish were handled and treated in accordance with guidelines approved by the U.S. Fish and Wildlife Service.

2.2. Fish culture

Rainbow trout eggs were obtained from Troutlodge Inc., Sumner, Washington, USA. They were cultured at the Bozeman Fish Technology Center, Bozeman, MT before they were randomly selected and stocked into 36 experimental tanks. Fish with an average initial weight of 15.7 ± 0.4 g were stocked at the rate of 20 fish/tank in a recirculating system, with three replicate tanks per dietary treatment. The culture water temperature was maintained at 15 ºC. Fish were acclimatized to tanks for one-week prior to beginning the feeding trial. Diets were randomly assigned to each of the tank and fish were fed twice a day to apparent satiation for the period of the experiment.

2.3. Diet manufacturing

All ingredients for each of the diets were ground with an air swept pulverizer (model 18-H; Jacobson, Minneapolis, MN, USA). All the diets were manufactured using cooking extrusion (DNDL-44, Buhler AG, Uzwil, Switzerland) with an 18-s exposure to an average of 127 ^{o}C in the extruder barrel section. The die plate was water cooled to an average temperature of 60 ^{o}C. Depending on the diet, pressure at the die head was varied from 15 to

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30 bar. The 3 mm pellets produced were then dried in a pulse-bed drier (Buhler AG, Uzwil, Switzerland) for 25 min at 102 ºC with a 10-min cooling period. The final moisture content in all the diets was <10%. Thereafter, oil was added to the diets by vacuum assisted top-coating (A & J Mixing, Ontario, Canada).

2.4. Growth monitoring and compositional sampling

During the 12-week growth trial, fish were weighed monthly and feed intake, feed conversion ratio (FCR) and weight gain were determined per tank. Ten fish from the initial fish population were sampled for determination of initial whole-body proximate composition. At the end of the 12-week feeding trial, three fish from each tank were sampled for determination of whole body composition. Three additional fish were sampled from each of the tank for determination of visceral somatic index (VSI), hepatosomatic index (HSI) and filet ratio (FR). Equations used to determine the body indices are displayed in Table 2.

Feces from fish in each replicate tank post sampling were obtained by manual stripping (Austreng, 1978). In brief, all fish in each tank were netted, anesthetized with MS-222 (Tricane methane sulfonate, Western Chemical Company, Ferndale, Washington, USA), dried and gentle pressure was applied to the lower abdominal region to express fecal matter into a plastic weighing pan. Care was taken to exclude urinary excretions from the collection. Fecal samples for a given tank were freeze-dried, ground with a mortar and pestle, and stored at -20 ^{o}C until chemical analyses (described below) were performed. Apparent dietary digestibility coefficients of dry matter, protein and energy were calculated according to established equations (Kleiber, 1961; Forster, 1999):

$$\text{ADCNdiet} = 100 \times \frac{(\text{\% marker in diet} \times \text{\% nutrient in feces})}{(\text{\% marker in feces} \times \text{\% nutrient in diet})} \quad (1)$$

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2.5. Histological studies

Following the 12-week feeding trial, GIT samples were obtained from three randomly selected fish per tank. Fish were euthanized and the digestive system was dissected to obtain the distal section of the GIT. Samples were preserved in Davidson's solution prior to histological evaluation. The distal intestine was examined using the longitudinal and cross sections for each fish. Histological cellular changes and alterations in tissues related to inflammation, supranuclear vacuolization in mucosal epithelial cells, increased thickness of the lamina propria or connective tissue, stratum compactum, number of mucus cells, degeneration and necrosis of mucosal epithelial cells, and numbers of rodlet were evaluated in each of the fish tissues following the standard histological procedures (Sheehan and Hrapchak, 1983). Scored of the Cellular changes observed ranges on a scale of 0 – 4, with 0 = no changes; 1 = minimal; 2 = mild; 3 = moderate; and 4 = marked. In general, scores of 1 and 2 are considered histologically normal or cellular changes of no significance; 3 is transitional or intermediary, moderate cellular changes that may or may not be within the normal range depending on species, age, and sex of the fish; 4 is indicative of pathological lesions (Sheehan and Hrapchak, 1983).

2.6. Short chain fatty acids analysis

To better understand whether GIT short chain fatty acid levels were altered with increasing levels of GDDY, short chain fatty acid analysis was carried out to compare only samples from 0% GDDY and 75% GDDY diets. Samples from the proximal section of the GIT were collected from three randomly selected fish from each tank. The samples collected were rapidly placed in liquid nitrogen and stored at -20 °C until analysis. Organic acids of acetic acid, butyric acid, propionic acid, valeric acid, isobutyric acid, isovaleric acids and 1, 3-butanediol (as the internal standard, IS) were purchased from Sigma Aldrich (Sigma-

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Aldrich, St Louis, MO USA) and were of HPLC grade with >99% purity. All the organic acids were at 0.5% (w/v) solution and 20 µl of each were individually mixed together. A 1 µl of 0.07% (v/v) mixture of the organic acids was directly injected into the gas chromatography mass spectrophotometer (Agilent Santa, Clara, CA) and the relative response factor (RRF) of each of the volatile organic acid was determined according to Yang et al. (2001) using Eq. (2).

$$RRF_{VOA} = \left(\frac{A_{VOA}}{A_{IS}}\right) / \left(\frac{W_{VOA}}{W_{IS}}\right)$$

$$= \left(\frac{A_{VOA}}{W_{VOA}}\right) / \left(\frac{A_{IS}}{W_{IS}}\right) \quad (2)$$

where *A* denotes peak area and *W* denotes weight in milligrams.

The recovery rate of each of the volatile organic acids in the fish samples was determined by mixing 200 µl of each of the GIT sample with 10 µl of 0.5% (w/v) of the IS. Three replicates were assayed per GIT sample and 1 µl of the mixture was directly was injected into the GCMS. The recovery rate was calculated for each of the volatile organic acids. Content of volatile organic acid in the GIT sample was calculated using Eq. (3).

$$VOA\ \left(\frac{mg}{ml}\right) = \left(\frac{A_{VOA}}{A_{IS}}\right) \times \left(\frac{W_{IS}}{RRF_{VOA}}\right) \times \frac{1}{V} \quad (3)$$

where, *V* denotes the volume of GIT sample in milliliters, *A* denotes peak area and *W* denotes weight in milligrams.

2.7. DNA extraction and PCR amplification

Three fish were randomly selected from each tank and were euthanized by overdose of tricane methane sulfonate (MS-222; 200 mg/L water). Following euthanasia and within 10 minutes, fish were aseptically dissected and the midgut luminal content and mucosal scrape samples collected (Betiku et al., 2017). The samples were stored in sterilized tubes and snap

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frozen in liquid nitrogen. All samples were stored in -20 °C prior to DNA extraction. Extraction of total genomic DNA from samples collected from the midgut section of the GIT was carried out using MoBio PowerFecal DNA isolation kits (MoBio Laboratories, Inc., Solana Beach, CA) following manufacturer's protocols.

To better understand changes in the functional potential of the midgut microbiota in response to increasing levels of GDDY with corresponding poor feed utilization observed with fish fed the 75% GDDY diet, shotgun metagenomic sequencing was carried out to compare only samples from 0% GDDY and 75% GDDY diets. A total of 24 samples were sequenced as twelve luminal and twelve mucosal scrape samples. Extracted DNA was quantified using Qubit assay kit (Life Technologies) and quality checked with the BioTek microplate Readers and Spectrophotometers using the standard 260/280 absorbance ratios. Polymerase chain reaction was performed using the Illumina Nextera XT kit (Illumina, Inc., San Diego, CA) following the manufacturer's instructions. After tagmentation step, the Illumina i7 and i5 indexes were used to amplify DNA genome PCR success and quality were checked Epoch 2 microplate Spectrophotometer (BioTek, Winooski, VT). The Nextera XT PCR products were cleaned using the AMPure XP beads and quantified post cleaning. The pooled library was diluted to at 12.5 pM and sequenced on an Illumina Miseq using 150 cycle v2 kits to obtain paired 75 nt reads. The sequences obtained were trimmed using fastq_quality_trimmer (http://hannonlab.cshl.edu/fastx_toolkit/license.html). This was followed by separation of paired and unpaired reads using customized Perl scripts. By using SOAPdenovo2, high quality reads were assembled de novo into contiguous sequences (Luo et al., 2012). A total of 337,198,603 reads were assembled into contigs (n50 ranged from 129-247 nt) and annotated in Metagenomics RAST server (Meyer et al., 2008). Complete functional and taxonomic annotation data were downloaded and manually curated to remove non-microbial hits (i.e. reads potentially deriving from the host or diet, which included reads

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from the phyla Chordate, Arthropoda, Echinodermata, Mollusca, Porifera, and Streptophyta). Because the coverage information of contigs generated by the SOAPdenovo assembly were imputed to the MG-RAST analysis (MG-RAST: A Technical Report and Manual for Version 3.3.6 - rev. 1 section 4.2.1) quantitative assessments of genes were enabled.

2.8. Analytical methods

Dry matter and ash analyses of ingredients, diets and whole body samples were performed according to standard methods (AOAC, 1995). Crude protein (N x 6.25) was determined in ingredients, diets and feces by Dumas method (AOAC, 1995) on a Leco TruSpec N nitrogen determinator (LECO Corporation, St. Joseph, Michigan, USA). Total energy was determined by isoperibol bomb calorimetry (Parr 6300, Parr Instrument Company Inc., Moline, Illinois, USA). Lipid was determined by petroleum ether extraction using an Ankom XT10 (Ankom Technologies, Macedon, New York, USA).

2.9. Statistical analyses

Proc GLM of SAS version 9.4 (SAS Institute Inc., Cary, NC, USA) was used for factorial ANOVA, while the Tukey's means separations were used to determine differences within main effects. Effects were considered significant at $P<0.05$. Taxonomic and functional data were compared between 0% GDDY and 75% GDDY diets, and between luminal and mucosal samples collected from the midgut GIT section. Differences in species abundance and functional capacity between diets were determined using analysis of similarity (ANOSIM) and non- parametric multivariate ANOVA (Adonis) of the Vegan package (version 2.3-5, Department of Statistics, Iowa State University, Ames, IA, USA) in R v. 3.2.2 (R Development Core Team, 2006). Similarity percentage, SIMPER was used to determine the relative contributions of each species to average similarities of the diet groups. Pearson's

bivariate correlations between microbial data and short chain fatty acids from the proximal sections of the GIT were calculated using the rcorr function in R statistical software and determined to be significant when r > 0.4 and P were <0.05. Microbial data were then corrected for multiple testing using the Benjamini-Hochberg False Discovery Rate (FDR) in SAS 9.4. ANOVA (P<0.05) p-values for the analysed variables were reported and were corrected for false discovery rate [FDR (*Q*>0.05)] resulting from multiple testing and the results are reported to guide future decision.

3. Results

3.1. Growth performance, body condition indices, proximate composition

During the trial, fish survival ranged from 98-100% with no differences among the treatment groups. There was no significant effect of prebiotics (P=0.5704) or GDDY inclusion level (P=0.4965) or significant interactions among the prebiotics and GDDY inclusion level (P=0.2524) on trout growth (Table 2). Weight gain was not different between dietary treatments (P=0.4876) and there was no significant effect of interaction (P=0.4530). In contrast, feed intake increased with increasing level of GDDY (P<0.0001), but was not affected by prebiotics inclusion (P=0.5783). In addition, FCR also increased with increasing GDDY level (P<0.0001) but no effect was observed with prebiotics (P=0.4397). No significant difference on VSI, HSI and FR with increasing GDDY levels, prebiotic supplementation or their interaction on the body indices of rainbow trout fed these dietary treatments (Table 2).

Whole-body lipid, energy and protein observed ranged from 12.9-13.3%, 2448-2536 kcal/g and 17.7-17.9%, respectively (Table 2). No significant effect of prebiotic supplementation, GDDY inclusion level or significant interaction on response variables analyzed was observed (Table 2).

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3.2. Diet digestibility results

Analyzed proximate composition (g kg) of the experimental diets for crude protein, crude lipid, and gross energy were 492 g kg^{-1}, 194 g kg^{-1}, and 23.5 MJ kg^{-1} respectively for 0% GDDY; 463 g kg^{-1}, 193 g kg^{-1}, and 23.4 MJ kg^{-1} for 50% GDDY; 452 g kg^{-1}, 201 g kg^{-1}, and 23.6 MJ kg^{-1} for 75% GDDY diets and reflected formulation targets (Table 3). Apparent dry matter, crude lipid and protein digestibility coefficients (ADCs) were 78.5%, 91.3%, and 91.7%, respectively for the 0% GDDY; 68.8%, 88.4%, and 89.4% for 50% GDDY diet; and 71.7%, 87.6% and 90.1% for 75% GDDY diet. Inclusion of prebiotics did not alter digestibility for the dietary treatments (Table 3).

3.3. Histology

The findings observed in this study were consistent among fish from the same tank but varied between tanks consuming the same dietary treatment. Cellular changes observed in the fish, irrespective of diet, were within the normal range. There were no scores indicative of pathological lesions that were specific to tanks or treatment groups. In general, specific inflammation and increased width of connective tissue were seen in fish fed 75% GDDY diet (Fig. 1a) compared to fish fed the 0% GDDY (fishmeal) diet (Fig. 1b), but there was no significant difference in tissue inflammation, vacuolation, mucus cells, D/N ME, thick CT, and thick LP in fish fed the dietary treatments (Table 4). However, there was a significant interaction (P=0.0028) between prebiotics and GGDY on rodlet cells.

3.4. Response of the GIT midgut microbes to diet and prebiotic

In total, sequence reads belonging to 2,285 microbial species and encoding 4,565 unique, annotatable gene functions were identified. Good's estimates of coverage indicated

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that >98 ± 0.01% of the GIT microbial species and gene functions were captured. The trout GIT microbiota were mostly bacteria (52 ± 33%), but eukaryotes (12 ± 13%), viruses (18 ± 25%), and Archaea (0.01 ± 0.03%) were also detected. Overall, the taxonomy of 13% of sequences were unable to be inferred. These unassigned sequences were mainly observed in fish consuming the GDDY-based diet.

A greater number of microbial species were observed in the 75% GDDY diet (n=278 ± 491) than 0% GDDY diet (116 ± 155) and this reflected Jackknife estimates of total microbial diversity (R<0.05). ANOSIM and Permutational Multivariate Analysis of Variance (PERMANOVA) indicated a diet X mid-GIT effect on microbial β-diversity (ANOSIM R=0.27, Adonis P=0.001). However, microbial species in the GIT were not significantly affected by prebiotic inclusion (ANOSIM $R < 0.05$ $P > 0.1$; PERMANOVA $P > 0.4$) alone. The most discriminating and significant phyla separating diets, as identified by Similarity Percentage Analysis (SIMPER) were mostly Bacteria from the phyla *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Spirochaetes*, *Tenericutes* and *Verrucomicrobia*. Significant increases in the Fungal phyla *Ascomycota* and *Basidiomycota*, were detected in the GDDY diets. Although dominant phyla were consistent across diets, their relative abundances changed between the fishmeal-based diet (0% GDDY) and grain distiller-rich diets. Between the two dietary groups, a greater relative abundances of the phyla *Firmicutes*, *Bacteroidetes* and *Spirochaetes* were seen in the 0% GDDY diet, while *Proteobacteria*, *Ascomycota*, *Basidiomycota*, and *Actinobacteria* were enriched in the GDDY diets (Figs. 2a and b).

3.5. Amino acids metabolism is affected by protein source and differed by GIT location

Shotgun metagenomic sequencing of samples of fish fed the 0% GDDY and 75% GDDY diets revealed Pearson's bivariate correlations among the microbial population and

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genes involved in transcription, and the metabolism of protein and several amino acids (Fig. 3). Specifically, positive correlations were observed among protein metabolism, protein biosynthesis; protein degradation; the metabolisms of histidine; lysine, threonine, methionine and cysteine; alanine, serine and glycine; transcription; RNA processing and modification; protein folding; protein processing and modification; and selenoprotein genes (Fig. 3). By contrast, genes involved in proteolytic pathways were negatively correlated with all these aforementioned genes excluding transcription and with *Acinetobacter* spp. (r=-0.64, P=0.03), and the yeast *Naumovozyma* spp. (r=-0.59, P=0.045). Proteolysis was also positively correlated with *Rhodococcus* spp. (r=0.59, P=0.04). Between the two diets, there was a 1.5 to 2-fold increase in genes associated with BCAA biosynthesis (P=0.028), aromatic amino acids (P=0.047), proteolytic pathways (P=0.0617), glycine biosynthesis (P=0.0173), thiamine biosynthesis (P=0.055), lipid-derived mediator (P=0.0072), coenzyme A (P=0.0071), urea decomposition (P=0.0457), and n-phenylalkanoic (P=0.0132) in 75% GDDY diet (Fig. 4). The relative abundance of isoleucine degradation gene (P=0.0085) was increased by 2-fold in same diet compared to fishmeal-based diet.

Genes involved in amino acid degradation had a greater relative abundance among mucosally-located microbes than those of the lumen. The relative abundance of genes related to BCAA (P=0.0299) and aromatic amino acids (P=0.0094) did not vary in luminal samples, but were approximately 2-fold greater in mucosa samples of fish consuming the 75% GDDY diet. These gene functions were also seen to correlate with *Acinetobacter* spp. (r>0.73 P<0.002). Similarly, there was a 2-fold greater relative abundance of genes involved in isoleucine degradation (P=0.0184) in mucosal samples of fish fed the 75% GDDY diet, compared to those fed the 0% GDDY diet. The proteolytic pathway, which comprises genes related to regulatory inter-membrane proteolysis pathway (P<0.0001) had a lower relative abundance in luminal samples than observed in mucosal samples of fish consuming the 75%

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GDDY diet, while the reverse was observed in the microbes of fish fed 0% GDDY diet. In contrast, an interaction of diet x mid-GIT sections effect for two-related proteases gene were lower in the luminal microbes of fish fed 75% GDDY, but had a 2-fold greater relative abundance in the mucosal samples of fish fed same diet; coenzyme A genes (P=0.0002) followed the same trend in the same diet. Also, an interaction effect for lipid-derived mediator (P=0.0003) was increased by 2-fold in the luminal microbes of fish fed 0% GDDY diet, while microbes showed a drastic reduction for the same gene in fish fed 75% GDDY. Furthermore, there was about 2-fold increase in GDH (P=0.0192) genes in both the luminal and mucosa microbes of fish fed 75% GDDY diet, while a significantly low level of this gene was observed in the GIT locations of fish fed 0% GDDY diet. There was a 2-fold increase in the fish fed 75% GDDY diet of genes associated with fermentation (P=0.0466), iron acquisition metabolism (P=0.0087), membrane transport (P=0.0001), aspartate aminotransferase (P<0.0001), translation (P=0.0017), murein hydrolases (P=0.0039), fructoooligosaccharides, and raffinose utilization (P=0.0001), heme uptake and utilization system (P<0.0001), creatine and creatinine degradation (P=00204), manganese transport P=0.0029), ABC transport of BCAA (P=0.0047), threonine anaerobic catabolism gene cluster, and nitrate and nitrite ammonification gene, but reverse effect was observed in these genes from the fish fed 0% GDDY diet.

3.6. Short chain fatty acids

Concentrations of Acetic (P=0.1994), propionic (P=0.8037), butyric (P=0.6268), valeric (P=0.5877) and isovaleric (P=0.5919) acids in the mid-GIT did not differ among the dietary treatments and were not affected by prebiotics supplementation (acetic, P=0.5117; propionic P=0.9608; butyric, P=0.5617; valeric, P=0.6549; and isovaleric, P=0.4064). No interaction of GDDY inclusion level and prebiotic supplementation were observed for acetic

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(P=0.0637), propionic (P=0.3680), butyric (P=0.5168), valeric (P=0.5381), and isovaleric (P=0.5778) acids (Fig. 5). However, Pearson correlation analysis indicated potential relationships between each of the Cyanobacterial phylum and acetic acid (r=0.683, P=0.042), the Actinobacteria phylum and propionic acid (r=0.444, P=0.03), and the Spirochetes phylum and valerate (r=-0.414, p=0.08). The relationship between Actinobacteria and propionic acid appeared to be mostly driven by Rhodococcus *spp.* (r = 0.78, P=0.003). While the bacterial genus *Escherichia coli* was found to correlate with butyric and isovaleric acids (r >0. 86, P<0.001). No other significant correlations were determined.

4. Discussion

The use of alternative ingredients as protein sources in rainbow trout diets has received considerable attention (Gomes et al., 1995; Kaushik et al., 1995; Lanari et al., 1998; Gaylord et al., 2007). Consistent and confounding effects of alternative diets in trout include increased feed intake and growth retardation when high quantities of certain ingredients are used in replacing fishmeal (Gomes et al., 1995; Adelizi et al., 1998; Cheng et al., 2003; de Francesco et al., 2004; Burr et al., 2012; Hauptman et al., 2014a; Hauptman et al., 2014b; Sealey et al., 2015b). However, significant improvements have been made on utilization of alternative protein diets when additional crystalline amino acids are added to balance dietary amino acid profiles to meet the tissue requirements of the fish (Davies and Morris, 1997; Gaylord et al., 2007; Gaylord and Barrows, 2009). The present study evaluated the ability of MOS and GroA prebiotics to attenuate any negative growth impacts on rainbow trout fed increasing amounts of GDDY-based diets (up to 75% GDDY) supplemented with free amino acids and compared with a conventional fishmeal-based diet (0% GDDY). The feed ingredients incorporated in the present study were the same as those used by Hauptman et al. (2014a; 2014b), but at different inclusion levels. In the earlier studies, increased levels of

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GDDY significantly reduced growth of trout and affected feed intake (Hauptman et al., 2014a; Hauptman et al., 2014b). However, in the present study, there were no significant growth effects of fishmeal replacement with GDDY up to 75%. This observation agrees with Gause and Trushenski (2011), who suggested that GDDY could replace up to 75% of protein from fishmeal in sunshine bass. Correspondingly, no positive effects on growth efficiency were observed when MOS and GroA were included in the GDDY diets or the fishmeal-based diet. The amino acid profile of the GDDY diets used in the present study were expected to meet the nutrient requirements of rainbow trout because the diets were formulated using the digestible amino acid composition of the GDDY protein. Moreover, since no negative effect resulted from the diet on the absorptive capacity of rainbow trout, based on histological observations and the comparable growth performance between fishmeal- and yeast-based proteins, GDDY has potential as a protein source in rainbow trout, which can replace fishmeal protein up to 75%. It is important to note that nucleic acid-based nitrogen is a substantial component of the yeast-based protein from *S. cerevisiae* used in this study (12-20%) and understanding the role of nucleic acids to rainbow trout nutrition is still in its infancy (Li and Gatlin, 2006). Our findings agree with earlier reports indicating that suggest nucleic acids are well utilized by rainbow trout (Rumsey et al., 1991; Rumsey et al., 1992), but further work is necessary to completely understand nucleic acid metabolism in fishes. The ability of yeast to act as an immuno-stimulant has been observed in cultured-fishes (Burrells et al., 2001; Ortuño et al., 2002; Leonardi et al., 2003; Li and Gatlin, 2004). Feeding GDDY, a yeast-based protein from *S. cerevisiae* in this study resulted in a 2-fold increase in genes related to microbial fermentation in fish fed the 75% GDDY diet. This supports the previous assertion that *S. cerevisiae* can aid fermentation for fast energy production (Otterstedt et al., 2004; Merico et al., 2007).

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Greater feed intake and lower feed efficiency observed in fish fed increased levels of GDDY, as observed in this study, are in line with what have been previously reported (de Francesco et al., 2004; Hauptman et al., 2014a; Hauptman et al., 2014b; Sealey et al., 2015b). Differences in nitrogen metabolism or appetite stimulation due to the addition of crystalline amino acids from the diets may be the culprit for the increased feed intake and poor feed efficiency associated with this ingredient (Martin et al., 2003; Snyder et al., 2012). However, a hyperphagic effect from amino acids supplementation on the GDDY diets was ruled out since there was no such effect observed in fish fed the fishmeal-based diet.

Midgut SCFA concentrations did not vary, indicating microbial fermentation in this region was not altered by dietary treatments. Since the midgut concentrations of acetic, propionic, butyric, isobutyric, valeric and isovaleric acids were not different in the trout fed either fishmeal or GDDY-based diets, it is unlikely that the poor feed utilization observed in the GDDY diet were driven by differences in microbial fermentation. Hence, another mechanistic explanation for the reduced feed utilization in trout was investigated.

From the metagenomics result, it was hypothesized that increased amino acid catabolism resulting from genes related to GDH cluster affected the efficient utilization of the absorbed nutrients. Due to the complexity of the amino acid pathways, more studies are needed to identify the contribution of other catabolic enzymes to amino acids catabolism and how environmental factors that are important in aquaculture, influence these processes to trigger changes in dietary amino acid utilization. Protein metabolism in rainbow trout involves dietary protein degradation and catabolism of amino acids in both liver and the small GIT tissues for synthesis of new proteins as well as other metabolites (Windmueller, 1982). In this study, greater numbers of gene clusters related to BCAA, aspartate amino transferase (AAT) and GDH activities in fish fed yeast-protein diet were observed, while the relative abundance of genes related to proteolysis decreased in the same fish. This observation is

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corroborated by earlier findings in that increased GDH and AAT enzyme activities are indicative of metabolic changes in pathways involved in the dietary utilization of alternative proteins in rainbow trout (Martin et al., 2003; Wacyk et al., 2012). Taken together, results of this study suggest catabolism of L-amino acids by AAT to glutamate increased the activity of GDH, a distinct adaptive response of amino acids deaminase enzyme to dietary protein levels (Cowley and Schmidt, 1995). This catabolic enzyme is up-regulated in microbes of fish consuming the GDDY-based diet, possibly increasing hepatic activity in the microbes for effecting the disposal of resulting amino acids from the GDDY-based diet via transdeamination. Although the contribution of nucleic acids to amino acids pool is not yet defined, an amino acid imbalance possibly from the nucleic acid content of the GDDY-based diet is suspected, which increased concentrations of GDH in fish fed this yeast diet compared to the fishmeal-based diet.

Alternatively, higher absorption rate of dietary glutamate from the yeast diet may be responsible for the increased GDH enzyme activity observed in the microbial genes of fish fed this diet. Similar changes in metabolic enzymes have been reported in rainbow trout fed dietary nucleotides (Keyvanshokooh and Tahmasebi-Kohyani, 2012). One explanation for this observation may be related to different time of appearance of essential amino acids and non-essential amino acids (Calbet and MacLean, 2002), an observation that is associated with either a plant protein diet (Larsen et al., 2012) or fast absorption of free amino acids (Schuhmacher et al., 1997) in rainbow trout. Although these observations require further investigation and special attention on the BCAA concentration is required to determine their role in this cascade of reactions involving GDH in rainbow trout, especially when alternative protein diets are used. Increased degradation of isoleucine cluster in the GIT microbes is of great interest, but why isoleucine is degraded in this study cannot be ascertained. A correlation between increased concentrations of circulating BCAA (Snyder et al., 2012) or

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glutamate (Burrin and Stoll, 2009) and overeating have been suggested. Possible explanations could be signals related to feed intake in the central nervous system that is regulated by changes in the concentration of circulating free amino acids (Leung and Rogers, 1973). This observation also requires further investigation.

Differences in feed utilization related to amino acid catabolism were seen in rainbow trout fed yeast-based diet. Greater numbers of microbial genes related to glutamate dehydrogenase observed in fish fed the yeast-based diet suggest microbes were competing for dietary amino acids resulting in poor utilization of high levels of GDDY in rainbow trout. The importance of the catabolic products to the host and the GIT microbiota is yet to be fully understood from the current study and should be investigated in future studies.

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Acknowledgements

OCB gratefully acknowledged Schlumberger Foundation for Faculty of the Future Fellowship. Funding for the study was provided, in part, by the Western Regional Aquaculture Center [grant numbers 2010-38500-13198, 2011-38500-14698, 2012-38500-15812, and 2013-38500-17048] from USDA-NIFA (PI: WMS). This study was funded by USDA-NIFA [grant number 2014-67004-2163] (PI: CJY), and a Montana Institute on Ecosystems award (PI: CJY). Authors wish to acknowledge technical assistance offered by Zachariah B. Conley and Sarah Olivo.

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Figure Legends

Fig. 1. Distal intestine of rainbow trout. (a) Trout fed 75% GDDY diet, (b) Trout fed 0% GDDY diet (control). LP = Lamina Propria.

Fig. 2a. Relative abundance of microbial populations in the mid-GIT of trout fed fishmeal and GDDY-based diets: 0% GDDY (control); 75% GDDY.

Fig. 2b. Relative abundance of microbial populations in the mid-GIT of trout fed fishmeal and GDDY-based diets: 0% GDDY (control); 75% GDDY with prebiotics inclusion.

Fig. 3. Correlation plot of microbial genes related to protein metabolism from mid-GIT of rainbow trout. A: Protein metabolism; B: Lysine, threonine metabolism; C: Alanine, Serine; D: Protein degradation; E: Protein biosynthesis; F: Proteolytic pathway; G: Transcription; H: Protein folding; I: Protein processing; J: Selenoprotein; K: RNA processing; L: Histidine metabolism.

Fig. 4. Fold increase of microbial gene functions affected by protein sources in rainbow trout. BCCA=Branched chain amino acid, Cys Biosynthesis=Cysteine Biosynthesis, Proteolytic path=Proteolytic pathway, GDH=Glutamate Dehydrogenase, Ile degradation=Isoleucine degradation, Urea decomp=Urea decomposition, Pentose P pathway=Pentose phosphate pathway, Asp aminotrans=Aspartate aminotransferase, Thiamine biosyn=Thiamine biosynthesis, Aromatic AA=Aromatic amino acids, Lysine Biosyn=Lysine Biosynthesis.

Fig. 5. Concentration of organic acids from mid-GIT samples of rainbow trout fed either 0% GDDY diet (control) or 75% GDDY diet. Means are from three replicate samples.

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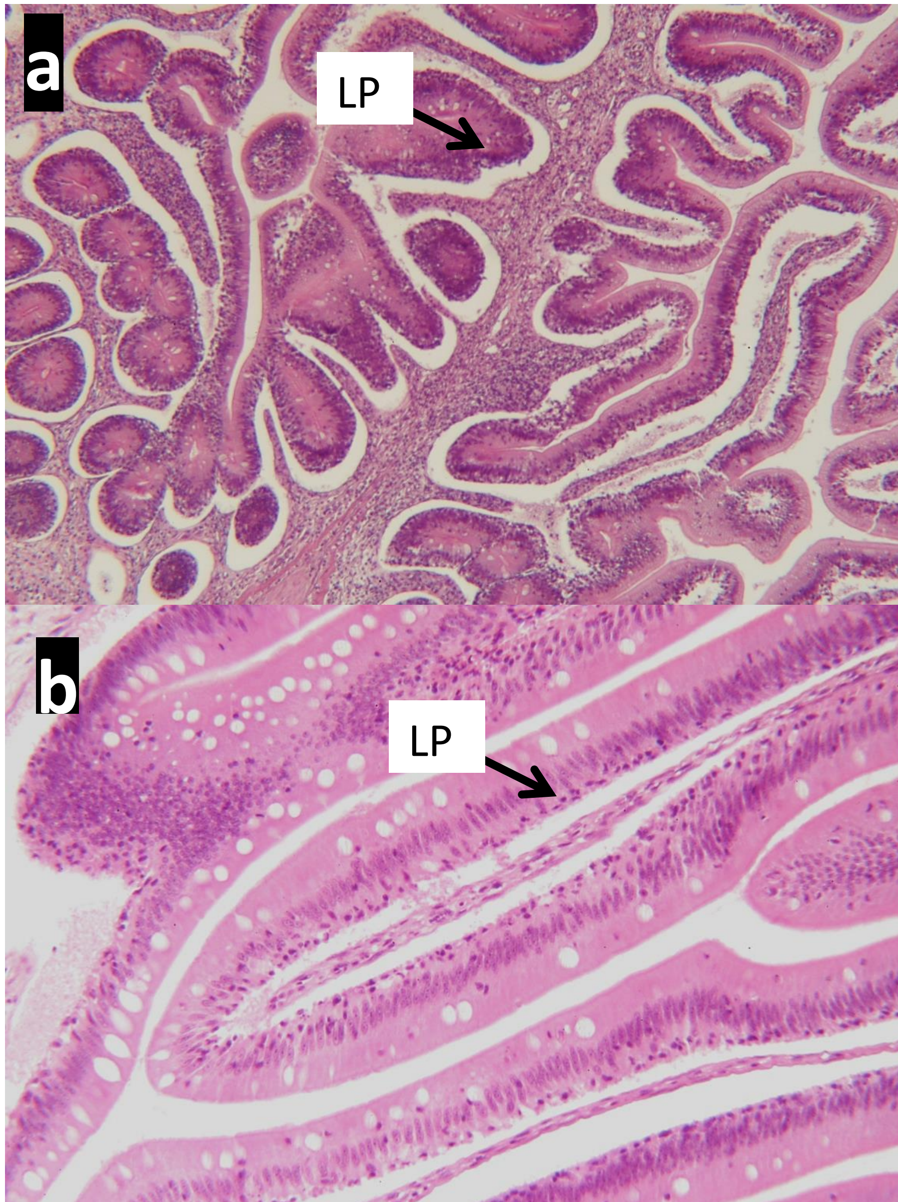

Fig. 1.

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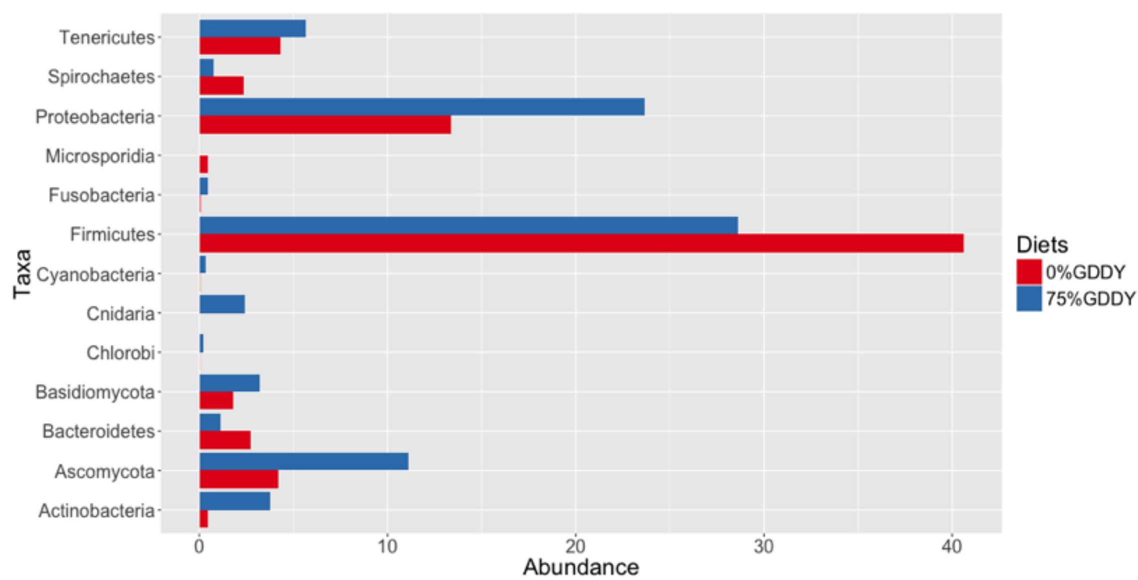

Fig. 2a

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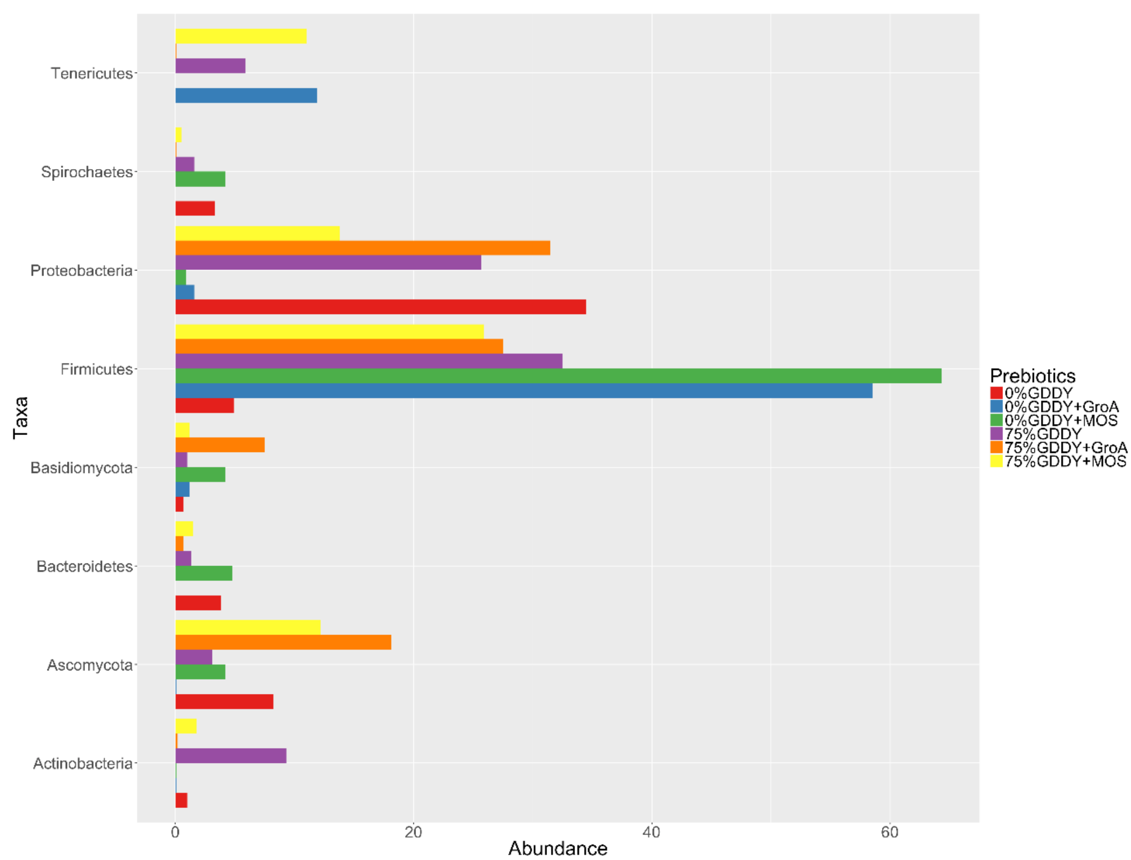

Fig. 2b

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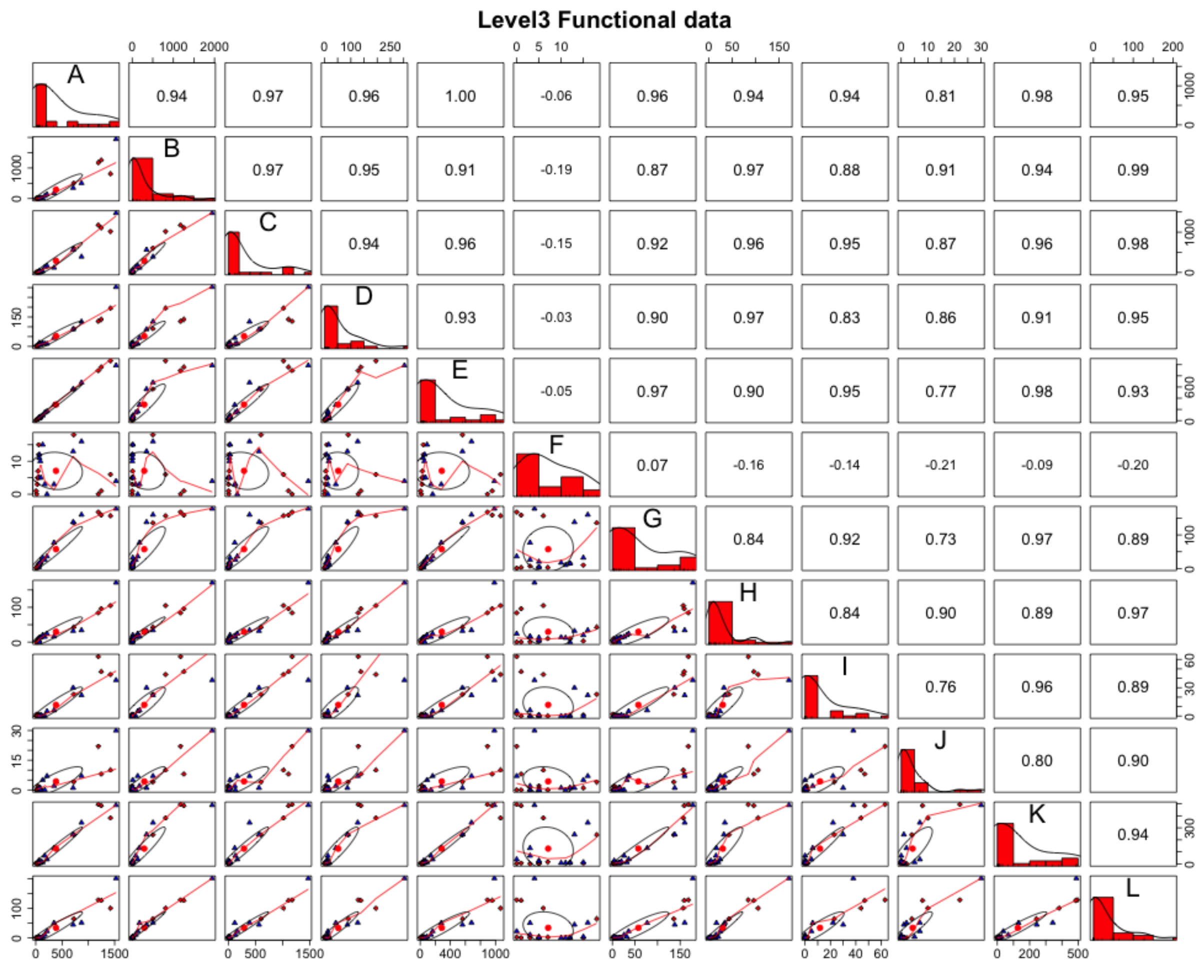

Fig. 3.

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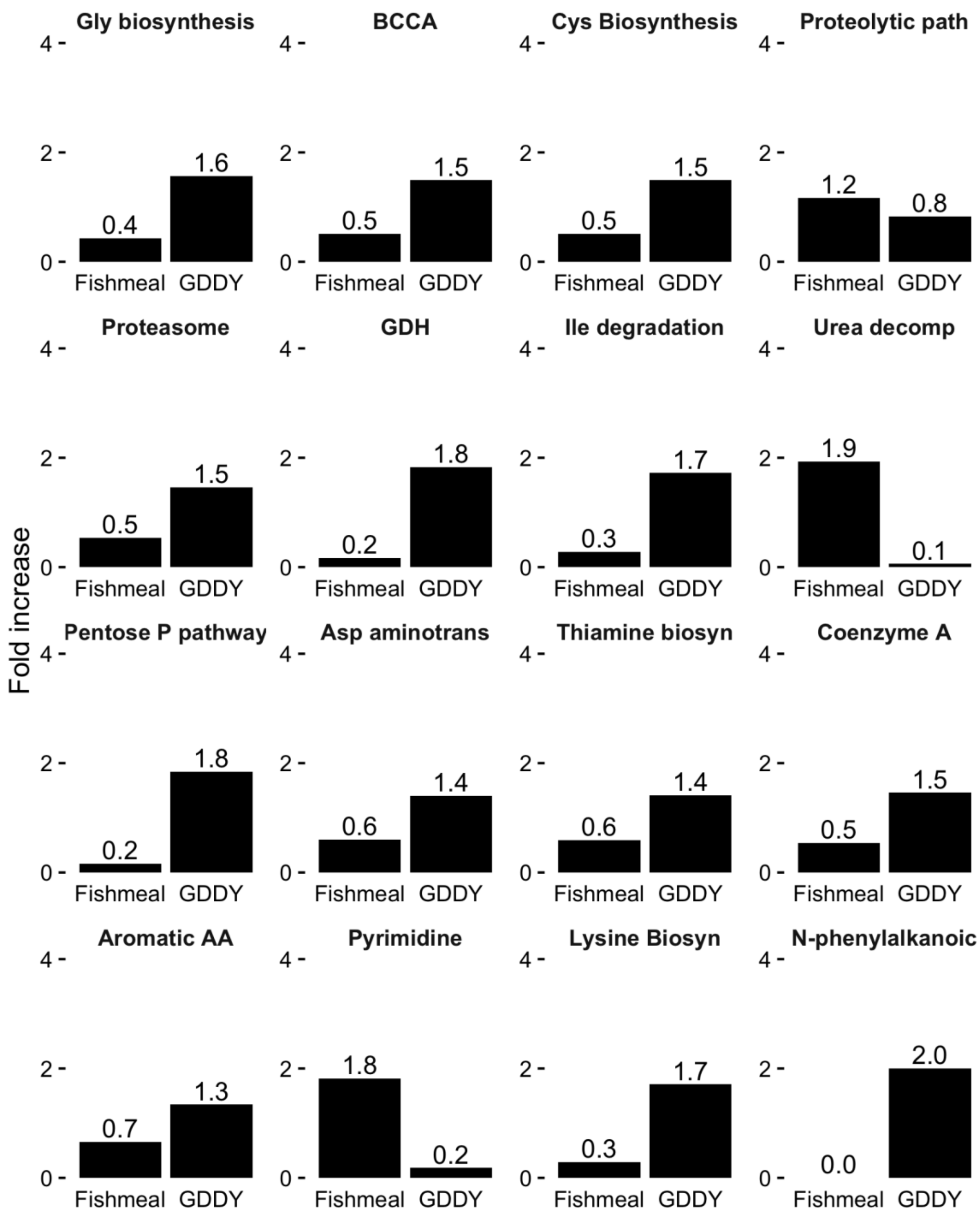

Fig. 4.

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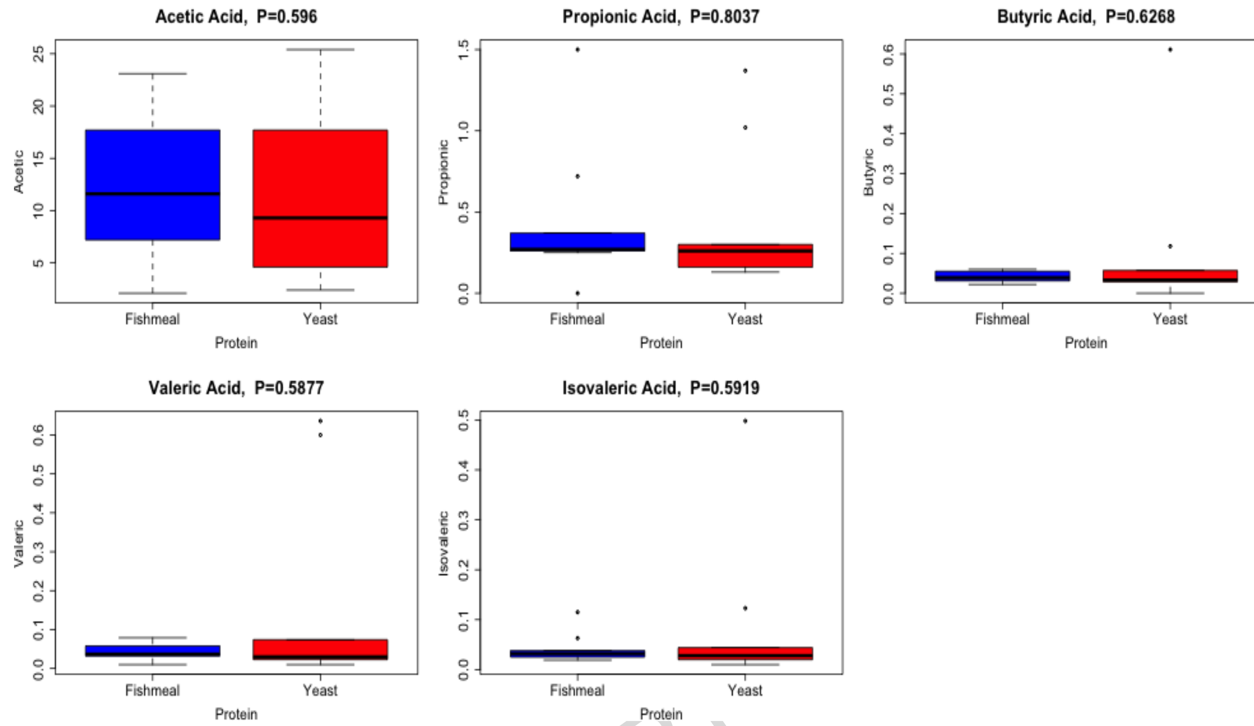

Fig. 5.

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Table 1. Ingredients compositions of dietary treatments.

Ingredient	Diets[1]											
	0% GDDY				50% GDDY				75% GDDY			
	0%	0.4% MOS	1% MOS	1% GroA	0%	0.4% MOS	1% MOS	1% GroA	0%	0.4% MOS	1% MOS	1% GroA
Grain distiller's dried yeast[2]	0	0	0	0	16	16	16	16	26.5	26.5	26.5	26.5
Menhaden fish meal[3]	25	25	25	25	12.5	12.5	12.5	12.5	6.25	6.25	6.25	6.25
Grobiotic A[4]	0	0	0	1	0	0	0	1	0	0	0	1
MOS[5]	0	0.4	1	0	0	0.4	1	0	0	0.4	1	0
Corn protein concentrate[6]	6.22	6.22	6.22	6.22	6.22	6.22	6.22	6.22	4.50	4.70	4.50	4.50
Soybean Meal 48% CP[3]	5	5	5	5	5	5	5	5	5	5	5	5
Soy protein concentrate[3]	5	5	5	5	5	5	5	5	5	5	5	5
Blood meal US[7]	4.85	4.85	4.85	4.85	4.85	4.85	4.85	4.85	4.85	4.85	4.85	4.85
Poultry by-product, pet food[7]	4	4	4	4	4	4	4	4	4	4	4	4
Wheat flour[7]	19.4	19	18.4	18.4	16.15	15.75	15.14	15.14	14.24	13.66	13.24	13.24
Menhaden fish oil[3]	18.03	18.03	18.03	18.03	18	18	18	18	17.6	17.6	17.6	17.6
Lecithin	1	1	1	1	1	1	1	1	1	1	1	1
Stay-C 35	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Vitamin premix ARS 702[8]	1	1	1	1	1	1	1	1	1	1	1	1

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TM ARS 640[9]	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
NaCl	0.28	0.28	0.28	0.28	0.28	0.28	0.28	0.28	0.28	0.28	0.28	0.28
Magnesium oxide	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06
Potassium chloride	0.56	0.56	0.56	0.56	0.56	0.56	0.56	0.56	0.56	0.56	0.56	0.56
Monocalcium phosphate	4.2	4.2	4.2	4.2	3.4	3.4	3.4	3.4	2.9	2.9	2.9	2.9
Choline Cl 50%	1	1	1	1	1	1	1	1	1	1	1	1
DL-Methionine	0.54	0.54	0.54	0.54	0.63	0.63	0.63	0.63	0.68	0.67	0.68	0.68
Lysine HCl	2.19	2.19	2.19	2.19	2.6	2.6	2.6	2.6	2.77	2.77	2.77	2.77
Threonine	0.74	0.74	0.74	0.74	0.82	0.82	0.83	0.83	0.88	0.87	0.88	0.88
Taurine	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Yttrium oxide	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Astaxanthin	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08

[1] Percent of fishmeal replaced by grain distiller's dried yeast on a digestible protein basis.
[2] Archer Daniels Midland (Decatur, IL, USA).
[3] Nelson & Sons Inc. (Murray, UT, USA).
[4] International Ingredient.
[5] Alltech, USA.
[6] Gavilon LLC, (Omaha, NE, USA).
[7] MGP Ingredients, Inc. (Atchison, KS, USA).
[8] Contributed per kg of diet: vitamin A (as retinol palmitate), 30,000 IU; vitamin D_3, 2160 IU; vitamin E (as DL-%-tocopheryl-acetate), 1590 IU; niacin, 990 mg; calcium pantothenate, 480 mg; riboflavin, 240 mg; thiamin mononitrate, 150 mg; pyridoxine hydrochloride, 135 mg; menadione sodium bisulfate, 75 mg; folacin, 39 mg; biotin, 3 mg; vitamin B_{12}, 90 µg.
[9] Contributed in mg/kg of diet: zinc, 37; manganese, 10; iodine, 5; copper, 3; selenium, 0.4.

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Table 2. Production performance metrics, body condition indices and proximate composition of rainbow trout fed yeast-based protein.

Diet	Weight gain[3] (% increase)	Feed intake[4] (% bw/d)	FCR[5] (g feed/g gain)	Visceral index[6] (%)	Fillet ratio[7] (%)	Hepatosomatic Index[8]	Body fat (%)	Body energy (cal/g)	Body protein (%)
0% GDDY+0% Pre	3208.1	1.9[c]	0.8[c]	32.3	63.7	2.6	13.0	2394.9	17.7
0%GDDY +0.4%MOS	3262.6	2.0[c]	0.8[c]	34.0	63.5	2.9	13.0	2443.6	17.5
0%GDDY +1%MOS	3172.7	1.9[c]	0.8[c]	28.5	63.4	2.6	13.4	2470.5	18.0
0%GDDY +1%GroA	3158.7	1.8[c]	0.7[c]	29.4	62.6	2.6	13.0	2481.9	18.5
50%GDDY+0% Pre	2986.7	2.0[b]	0.8[b]	26.5	57.1	2.5	12.4	2368.3	18.0
50%GDDY+0.4%MOS	3116.3	2.0[b]	0.8[b]	34.9	62.5	2.7	12.6	2405.1	18.4
50%GDDY +1%MOS	3009.8	2.0[b]	0.8[b]	32.8	64.7	3.0	13.4	2534.9	18.0
50% GDDY +1%GroA	3120.7	2.0[b]	0.8[b]	34.1	53.0	2.6	13.4	2404.7	17.8
75%GDDY+0% Pre	2973.7	2.3[a]	0.9[a]	35.2	63.1	2.8	12.9	2533.9	17.9
75%GDDY+0.4%MOS	2795.4	2.3[a]	1.0[a]	32.6	55.4	2.5	13.4	2583.8	17.3
75%GDDY+1%MOS	3192.7	2.2[a]	0.9[a]	32.3	58.7	2.8	13.1	2519.8	17.9
75%GDDY+1%GroA	3413.7	2.3[a]	0.9[a]	38.4	63.5	3.0	12.3	2461.5	17.9
Pooled SEM	172.2	0.06	0.03	3.31	4.53	0.21	0.48	106.5	0.37
Main effect means									
GDDY	3117.6	2.1	0.8	32.5	60.9	2.7	12.9	2466.9	17.9
Prebiotic	3118	2.1	0.8	32.6	60.9	2.7	13	2466.9	17.9
ANOVA, Pr > F[2]	0.5858	<0.0001	<0.0001	0.5113	0.6960	0.7861	0.7597	0.9452	0.6145

[1] Initial tank weights were 15.7 ± 0.4 g with no significant differences between tanks, for all analyses means of three replicate tanks were presented (20 fish/tank).
[2] Mean determinations of nine fish per treatment; three replicate tanks per diet.
[3] Weight gain (%) = (final weight – initial weight)*100 / initial weight.
[4] Feed intake (%) = g dry feed consumed/average fish biomass (g) /culture days *100.
[5] FCR, feed conversion ratio = g dry feed consumed / g weight gained.[4] Weight gain (%) = (final weight – initial weight)*100 / initial weight.
[6] Visceral somatic index (%) = viscera mass x 100 / fish mass.
[7] Fillet ratio (%) = fillet with rib mass * 100 / fish mass.
[8] Hepatosomatic index (%) = liver mass x 100 / fish mass.

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Table 3. Diet proximate compositions and apparent digestibility coefficient[1].

Diet	Analyzed proximate composition			Apparent digestibility coefficient		
	Crude Protein (N*6.25)	Fat (g/kg)	Gross Energy (MJ/kg)	DM*	Fat*	Protein*
0%GDDY	492	194	23.5	78.5[a]	91.3[ab]	91.7[abc]
0%GDDY +0.4%MOS	495	210	23.4	76.6[abc]	92.5[a]	91.4[abc]
0%GDDY +1%MOS	488	198	23.4	76.0[b]	90.0[abcd]	91.9[ab]
0%GDDY +1%GroA	489	184	23.3	77.3[a]	92.1[abc]	92.1[a]
50%GDDY	463	193	23.4	68.8[bcd]	88.4[defg]	89.4[cd]
50%GDDY+0.4%MOS	461	203	23.7	68.0[cd]	89.7[bced]	88.8[d]
50%GDDY +1%MOS	460	198	23.5	69.0[d]	87.9[efg]	89.4[d]
50% GDDY +1%GroA	458	195	23.1	71.3[cd]	89.2[cdef]	90.3[cd]
75%GDDY	452	201	23.6	71.7[d]	87.6[fgh]	90.1[d]
75%GDDY+0.4%MOS	451	200	23.6	68.8[cd]	85.5[efg]	89.9[bcd]
75%GDDY+1%MOS	454	176	23.6	70.3[cd]	86.2[gh]	90.3[abcd]
75%GDDY+1%GroA	459	183	23.6	66.9[d]	84.4[h]	88.7[d]

[1] Means of duplicate analyses on a dry matter basis (mg g^{-1} sample). *Pr>F for DM (<0.0001), Protein (<0.0001), Fat (<0.0001).

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Table 4. Histological data of rainbow trout fed the dietary treatments.

Parameter	0% GDDY				50% GDDY				75% GDDY				P>F[2]
	0%	0.4% MOS	1% MOS	1% GroA	0%	0.4% MOS	1% MOS	1% GroA	0%	0.4% MOS	1% MOS	1% GroA	
Inflammation	2.25	1.80	2.60	1.75	2.25	1.80	1.20	1.50	2.25	1.40	2.40	2.25	0.5477
Vacuolation	1.50	2.20	2.40	2.00	1.75	1.25	2.20	1.75	1.50	1.80	2.00	2.25	0.4276
Mucus	2.50	2.80	2.60	2.67	2.25	2.25	2.40	2.50	2.75	2.60	3.00	2.75	0.6952
Cells	2.25	2.00	1.80	1.67	1.50	1.75	1.60	1.75	1.75	2.00	2.40	2.00	0.6497
DN	1.50	1.20	1.60	1.33	1.25	1.00	0.60	1.25	2.00	1.00	1.60	2.00	0.7446
Thickct	1.25	0.80	1.00	0.67	0.75	0.50	0.60	0.75	1.50	0.60	1.40	1.75	0.2003
Thicklp	1.25	2.40	1.20	1.67	1.50	1.00	1.60	2.00	1.50	2.0	1.80	1.50	0.2289
Rodletcells	27.00	18.00	20.20	30.50	26.25	23.40	20.40	22.75	23.25	18.20	22.00	25.00	0.9789

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Highlights

- GDDY supplemented with limiting amino acids replaced up to 75% fishmeal in trout diet.
- Feed utilization was affected in trout fed GDDY diet, but not growth.
- Some fungi and phylum *Actinobacteria* were enriched in trout fed GDDY diet.
- Genes associated with BCAA were elevated in trout fed GDDY diet than those fed fishmeal protein.
- Increased GDH gene clusters in GIT of GDDY fed fish affected amino acids utilization.

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