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Research Article

Analysis of the Gut Microbiome of Nile Tilapia *Oreochromis Niloticus* L. Fed Diets Supplemented with Previda® and Saponin

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Abstract: The use of prebiotics in aquaculture aims at increasing the numbers of beneficial microbes in the gastrointestinal tract of fishes. However, the true effect of prebiotics exert on the fish microbiome is for the most part unknown. This study aimed at investigating the effects of the commercial prebiotic Previda® and the feed supplement saponin on Nile tilapia (*Oreochromis niloticus L.*). We used 454 pyrosequencing to characterize the gut microbiome of fish fed diets containing Previda®, saponin, a combination of both, and a control diet. After data normalization, 98,300 sequence reads were analyzed resulting in 331 unique operational taxonomic units (OTUs). Three phyla were present in all samples: Fusobacteria, Bacteroidetes, and Proteobacteria. Fusobacteria was the predominant phylum in all samples accounting for more than 80% of the OTUs. Within the Fusobacteria, the predominant genus was Cetobacterium with more than 76% of the sequences being ascribed to that species. Barnesiella was the second most common genus with more than 6% of the total sequences. Overall, there was not a significant difference in the composition of the gut microbiomes regardless of the diet. The overwhelming numbers of Cetobacterium sp. present in the gut of Nile tilapia could overshadow the effect of the treatments on other genera.

Keywords: Gut microbiome; Tilapia; Saponin; Mannose oligosaccharide

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Introduction

The use of probiotics and prebiotics in aquaculture has increased tremendously in recent years as a sustainable alternative to the use of antibiotics. The main goal for administering probiotics (live microorganisms) or prebiotics (fermentation byproducts that promote the growth of beneficial microbes) is to manipulate the gut microbial community or gut microbiome to improve host fitness, mainly through the exclusion of opportunistic pathogens (Balcazar et al., 2006). In addition, the gut microbiome strongly influences fish health in other ways such as assisting in the development of the gut epithelium, providing essential nutrients, and stimulating the innate immune system (Nayak, 2010). Thus, alteration of the gut microbiome with probiotics or prebiotics may prevent disease through a variety of mechanisms. Until recently, the characterization of gut microbial communities in fish was biased due to the use of culture-dependent methods that vastly underestimated the species richness of the gut microbiome. The development of cost-effective next generation sequencing methods promoted the use of culture-independent methods resulting in detailed descriptions of these complex microbial communities. Nevertheless, the basal composition of the gut microbiome of important economic species such as Nile tilapia *Oreochromis niloticus* L. is still largely unknown. Several studies have analyzed the effects of different feed additives in the intestinal bacterial community of tilapia; however, those studies employed a fingerprint-based method (DGGE) suitable for identifying changes in the communities but without describing species composition (Pedrotti et al., 2015, He et al., 2011, Zhou et al., 2009, Liu et al., 2014).

Previda® is a commercial prebiotic composed primarily of mannose oligosaccharides that have been reported to improve growth, performance, and immunological responses in Pacific white shrimp (Anuta et al., 2014). Preliminary studies suggest Previda® also alters the gut microbiome in fish (Burr et al., 2012). Saponins are secondary plant compounds that have been tested in aquafeeds with different results. While some reports documented the toxic effect of saponin in fish and its negative effects to the gut epithelium (Knudsen et al., 2008), some studies concluded saponin promotes growth (Francis et al., 2005). Furthermore, saponin has been studied as a natural steroid to promote sex inversion to an all-male population, which could represent an alternative to the use of androgenic hormones in tilapia culture (Francis et al., 2002). The combined use of prebiotics such as Previda® that promotes intestinal health could offset the intestinal damages exerted by saponin. The objectives of this study were two-fold. First, we aimed at describing the gut microbiome of Nile tilapia fed standard diets using pyrosequencing. Second, we characterized the changes in the gut microbiome induced by feeding diets amended with Previda® and saponin.

Material and Methods

Diet preparation

Previda®, saponin, and their combination were supplemented as feed additives to a practical basal diet **(Table 1)**. The basal diet was formulated to meet the nutritional requirements of tilapia containing 32% protein and 6% lipid (Ng and Romano, 2013). The diets contained 4% fishmeal and 3.4% menhaden fish oil to ensure nutritional quality of the diets and to mask any potential palatability problems due to the addition of the supplements. Previda® and saponin were added to the basal diet at 0.5% and 0.3% inclusion levels, respectively. Concentrations were chosen based on previously published data that showed an effect of Previda® in the gut microbiome of shrimp (Anuta et al., 2014) and the benefits of saponin in Nile tilapia diets (Francis et al., 2005). Experimental diets were prepared at the fish nutrition laboratory at the E. W. Shell Fisheries Center, Auburn University, Auburn, AL (USA) using standard practices. Briefly, pre-ground dry ingredients and fish oil were mixed in a food mixer (Hobart Corporation, Try, OH, USA) for 15 minutes. Hot water was blended into the mixture and then pelleted through a 3 mm die using the same mixer. Pelleted diets were dried in an oven to a moisture content of 8-10%, bagged, labeled, and stored at 4°C until feeding.

Fish husbandry and experimental conditions

All male Nile tilapia juveniles (n=250, mean weight=4.10 ± 0.74 g; mean length=5.2 ± 0.3 cm) were obtained from E. W. Shell Fisheries Center at North Auburn Fisheries Experiment Station and transported to the Aquatic Microbiology Laboratory (AML) on main campus in aerated containers. Upon arrival to AML, fish were stocked into 37 L glass aquaria/tanks at a stocking rate of 12 fish/tank. Ten randomly-selected individuals were used to estimate weight and length and also to check for common fish pathogens according to standard protocols (USFWS and FS-FHS, 2012). Fish were acclimated for 10 days and fed the basal diet at 2% body weight daily. Each tank contained an individual biofilter and an air stone. Water was prepared with 340 g of Marine Salt (Seachem, Madison, Georgia) diluted in 10 L of deionized water to make the primary salt stock. For tank use, 0.6 g of CaCO3, 1.4 g of NaHCO3, 0.17 g NH$_4$Cl (5 ppm) and 90 ml of the stock were mixed into each tank with deionized water. Water quality was checked daily to maintain established parameters (80 ppm alkalinity, 40 ppm hardness, 0.1 ppt salinity, 27 ± 1°C, pH 7.8 ± 0.2 [mean ± standard error], ammonia and nitrites were non-detectable). After the acclimation period, tanks (5 replicates) were randomly assigned to each experimental diet (4 treatments). All treatment groups were fed at the same fixed rate of 2% body weight for two weeks. All animal protocols were approved by the Auburn University Institutional Animal Care and Use Committee.

Sample collection and DNA extraction

After two weeks of dietary conditioning, 10 fish were randomly removed from each tank and immediately euthanized with 300 mg/L tricaine methanesulfonate. Fish (n=200, mean weight=5.49 ± 0.14 g; mean length=5.3 ± 0.5 cm) were measured to observe possible changes in growth over the course of the experiment. The gastrointestinal tract from the posterior stomach to the anus was aseptically removed from each individual, and the gut content (feces) was squeezed from the intestine to be immediately processed for DNA extraction. Gut contents from

Table 1: Composition (g/100 g) of experimental diets designed to contain 32% protein and 6% lipid.

Ingredients	Diets			
	1 Basal	2 Previda®	3 Saponin	4 Previda®+Saponin
Menhaden fishmeal[1]	4.00	4.00	4.00	4.00
Soybean meal[2]	51.00	51.00	51.00	51.00
Corn protein concentrate[3]	5.10	5.10	5.10	5.10
Menhaden fish oil[1]	3.40	3.40	3.40	3.40
Corn[4]	31.00	31.00	31.00	31.00
Corn Starch[5]	0.90	0.40	0.60	0.10
Trace mineral premix[6]	0.50	0.50	0.50	0.50
Vitamin premix[7]	1.80	1.80	1.80	1.80
Choline chloride[5]	0.20	0.20	0.20	0.20
Stay C®[8]	0.10	0.10	0.10	0.10
CaP-dibasic	2.00	2.00	2.00	2.00
Previda®[9]	0.00	0.50	0.00	0.50
Saponin[10]	0.00	0.00	0.30	0.30

[1]Special Select, Omega Protein Inc., Reedville, Virginia, USA.

[2]De-hulled solvent extracted soybean meal, Faithway Feed Co. Inc., Guntersville, Alabama, USA.

[3]Empyreal 75, Cargil Corn Milling, Blair, Nebraska.

[4]Yellow corn, Faithway Feed Co., Guntersville, AL, USA.

[5]MP Biochemicals Inc., Solon, Ohio, USA.

[6] Trace Mineral premix\(g/100 g), cobalt chloride 0.004, cupric sulphate pentahydrate 0.250, ferrous sulfate 4.0, magnesium sulfate heptahydrate 28.398, manganous sulphate monohydrate 0.650, potassium iodide 0.067, sodium selenite 0.010, zinc sulfate heptahydrate 13.193, filler 53.428.

[7]Contained (as g/kg premix): thiamin-HCl, 0.438; riboflavin, 0.632; pyridoxine-HCl, 0.908; D-pantothenic acid, hemicalcium salt, 1.724; nicotinic acid, 4.583; biotin, 0.211; folic acid, 0.549; vitamin B12, 0.001; inositol, 21.053; menadione sodium bisulfite, 0.889; vitamin A acetate (500,000 IU/g), 0.677; vitamin D3 (1,000,000 IU/g), 0.116; DL-alpha-tocopheryl acetate (250 IU/g), 12.632; alpha-cellulose, 955.589.

[8]Stay C® (L-ascorbyl-2-polyphosphate 35% Active C), DSM Inc., Parsippany, NJ, USA.

[9]Previda®, Novus Nutrition Brands LLC, St Charles MO.

[10]Alfa Aesar, A Hohnson Matthey Compnay, Saponin, A18820 lot 10170564. Haverhill, MA.

10 individual fish (from the same tank) were pooled together into one sample for gut microbiome analysis. Pooling of samples was necessary to obtain sufficient material for DNA extraction. Each diet treatment group was thus represented by 5 replicates. DNA extraction followed previously published protocols (Larsen et al., 2014b). Briefly, gut content was homogenized using a handheld tissue homogenizer to ensure uniform sample distribution. DNA was extracted from samples of approximately 200 mg (Ave. 205 ± 21.5 mg) using the DNA isolation for pathogen detection protocol included in the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA). The manufacturer's protocol was used with only minor deviations from standard protocols. During the elution step (Step 17) of this protocol, only 50 µl of buffer AE was used rather than the suggested 200 µl to prevent excessive dilution of the DNA products (Qiagen 2001).

Sequencing

Roche titanium 454 sequencing was performed on 20 samples (5 replicates per diet treatment) using individual barcodes and primer 27F (5-AGRGTTTGATCMTGGCTCAG-3') amplifying the variable V1-V3 region of the 16S rRNA. PCR conditions were as follows: initial denaturation at 94°C for 3 min and 30 cycles of 94°C for 30 s, 53°C for 40 s, and 72°C for 1 min, including a final elongation at 72°C for 5 min. Resulting sequences were processed with an exclusive analysis pipeline (MR DNA, Shallowater, TX) including removal of barcodes and primers as well as sequences of less than 200 base pairs, a base call error rate of less than 0.3% (Q<25), ambiguous base calls, and long (>6 base pairs) stretches of identical bases. Following denoising along with chimera and singleton sequence removal, operational taxonomic units (OTUs) were defined in agreement with the current accepted prokaryotic species concept (>3% sequence agreement; (Rossello-Mora and Amann, 2001)) and taxonomically identified using BLASTn against the Greengenes database (DeSantis et al., 2006).

Data analysis

After standardization of each sample type to the sample yielding the least number of total sequences, Mothur v.1.33.3 (Schloss et al., 2009) was used to generate rarefaction curves and to calculate diversity statistics including Good's coverage, Shannon Evenness Index (SEI), abundance-based coverage estimation (ACE), Chao1, observed OTUs, and shared OTUs. SAS 9.2 was

used to run one-way ANOVAs with Tukey's multiple comparison tests (α=0.05) in order to determine differences in species richness (in observed OTUs) and species evenness (SEI) between samples (diet treatments). OTU tables including all samples were loaded into Primer v6 for clustering using group averages and ANOSIM analysis. Genera tables were also loaded into Primer for similarity percentages (SIMPER) analysis in order to determine specific taxonomical differences between communities.

Results

Water quality, growth and survival

All water quality parameters remained within established limits throughout the study. Diets containing saponin (both saponin-only and mixed treatments) resulted in fecal pellets of softer consistency when compared to controls. The Previda® treatment did not result in any obvious morphological changes of the fecal pellets. After two weeks of feeding, the average fish weight increased significantly (when all treatments were taken into consideration), but length remained statistically identical. Final averages for length and weight were 5.33 ± 0.51 cm and 5.49 ± 1.37 g, respectively. Neither final weight nor length was different between diet treatments. No mortalities were observed during the study.

Analysis of tilapia gut microbiome

A total of 281,349 bacterial sequences were obtained from the tilapia gut microbiome by pyrosequencing. Overall, 345 OTUs were identified from all 20 samples analyzed including four diet treaments with five replicates each. In order to accurately compare species richness and evenness, sequences were standardized to the lowest number of obtained sequences (n=4,915) among the 20 samples for a total of 98,300 sequences and 331 OTUs. Analysis of Good's coverage indicated sequence coverage was ≥ 98% for all samples **(Table 2)**. **Figure 1** shows the rarefaction curves obtained from all diet treatments. A good coverage of the gut microbiome was achieved in all cases regardless of the treatment. One-way ANOVA with Tukey's multiple comparison tests showed no significant differences between diet treatments, neither in the expected richness (ACE and Chao1) nor in evenness (SEI). All diet treatments exhibited relatively high overlap of OTUs with 45% of OTUs being shared between all four diets **(Figure 2)**. A similar number of unique OTUs present only in basal (n=20), Previda® (n=22), and saponin (n=18) diets were also identified. Interestingly, the mixed diet also resulted in gut microbiomes with unique OTUs (n=12).

Gut microbiome composition

Sequencing analysis revealed the gut microbiome of

Table 2: Diversity indices generated by Mothur software. OTUs are defined at 97% sequence similarity. B-Basal diet; P-Previda® diet; M-Mixed diet; S-Saponin diet.

Sample ID	# Observed OTUs	# Predicted OTUs		Shannon evenness	Good's coverage
		ACE	Chao1		
B1	139	190	182	0.595	0.991
B2	141	269	224	0.527	0.989
B3	128	173	161	0.552	0.991
B4	132	187	181	0.564	0.991
B5	130	226	167	0.551	0.991
P1	133	201	172	0.634	0.992
P2	118	190	151	0.543	0.992
P3	119	162	152	0.556	0.992
P4	133	226	201	0.568	0.99
P5	135	283	216	0.572	0.989
M1	135	185	189	0.555	0.991
M2	132	230	188	0.59	0.99
M3	149	285	229	0.605	0.988
M4	127	184	191	0.543	0.99
M5	121	233	178	0.55	0.99
S1	131	165	166	0.561	0.992
S2	127	182	186	0.513	0.99
S3	137	271	243	0.552	0.989
S4	146	261	216	0.587	0.989
S5	139	189	176	0.534	0.991
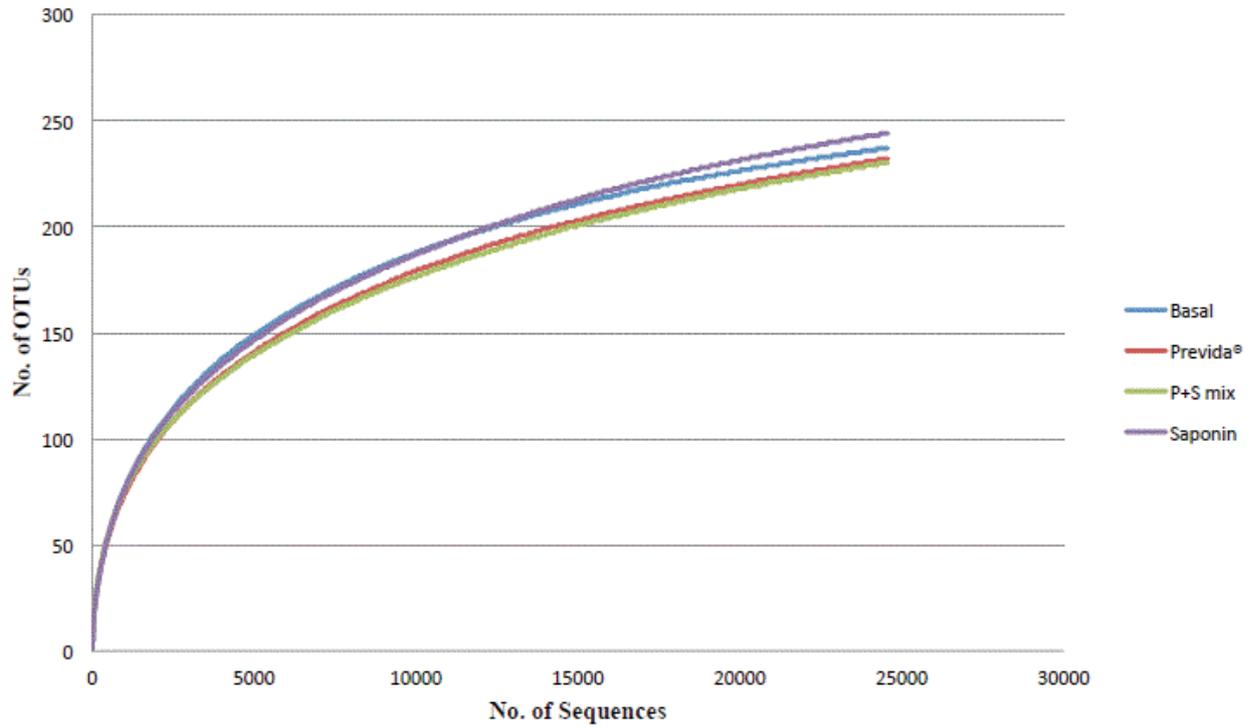
Figure 1: Rarefaction curves by diet. Sequences were standardized to equal sample sizes for direct comparison.

Comparison	Shared OTUs
B	237
P	230
M	232
S	244
B-P	178
B-M	185
B-S	187
P-M	184
P-S	187
M-S	193
B-P-M	159
B-M-S	165
P-M-S	167
B-P-S	160
B-P-M-S	149
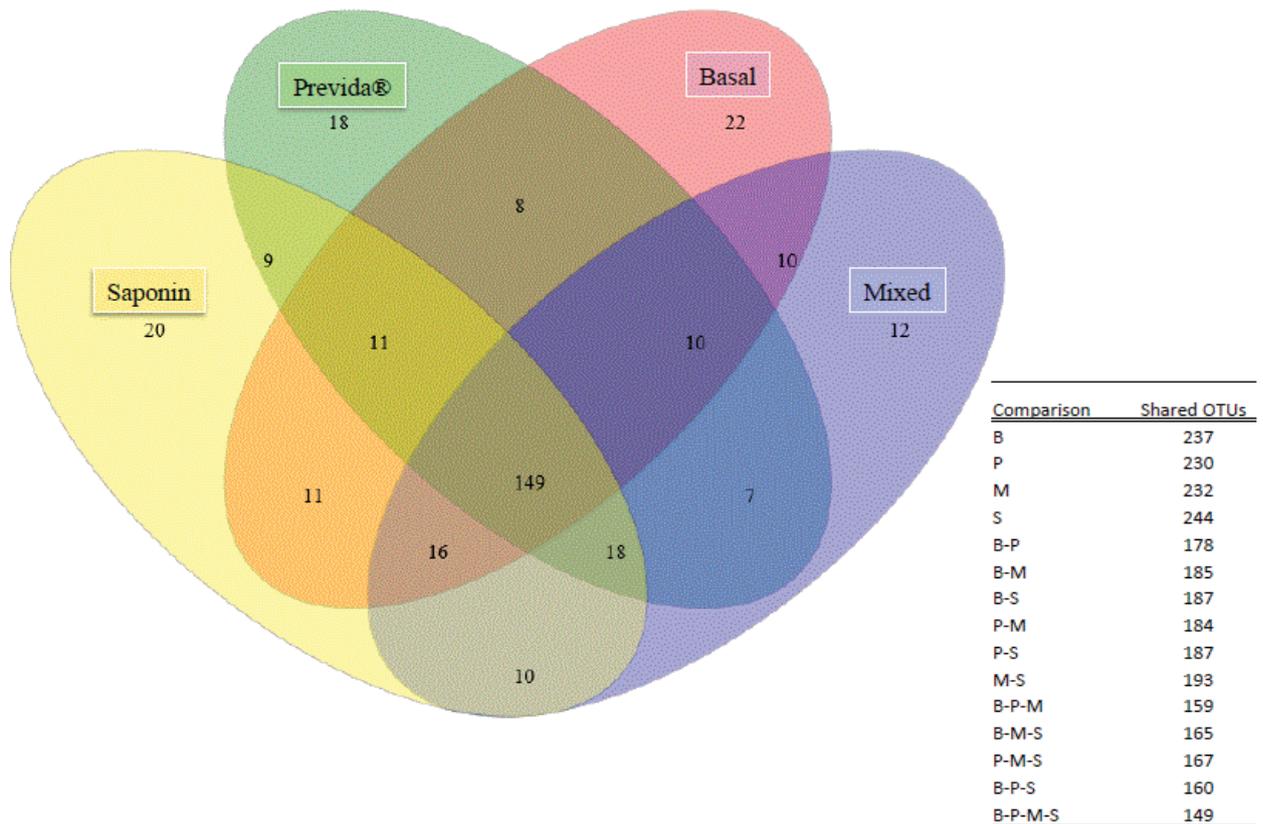
Figure 2: Venn diagram representing shared operational taxonomic units (OTUs) between diets.

tilapia was composed of six bacterial phyla (i.e., Fusobacteria, Bacteroidetes, Proteobacteria, Firmicutes, Planctomycetes, and Actinobacteria) **(Figure 3)**. Out of the six phyla, only three (Fusobacteria, Bacteroidetes, and Proteobacteria) were shared between all four treatment groups. While the relative abundances of these phyla varied somewhat between the four, all diets were individually dominated by the phylum Fusobacteria ($\geq 81\%$) followed by Bacteroidetes ($\geq 5.9\%$) and Proteobacteria ($\geq 0.1\%$). The three less common phyla, Actinobacteria, Firmicutes, and Planctomycetes, showed varying abundances between the four diets. Actinobacteria were found in all treatments with the exception of the saponin diet. Firmicutes and Planctomycetes were both found in the basal diet; however, Planctomycetes was unique to the gut microbiome associated with the mixed diet, while the phylum Firmicutes was unique to the gut microbiome of saponin fed fish.

Out of 26 identified genera, only five were found to be present among all four diet treatments (i.e. Barnesiella, Cetobacterium, Parabacteroides, Plesiomonas, and Pseudomonas) **(Table 3)**. The genus Cetobacterium was vastly represented and accounted for 90% of the total OTUs found in all analyzed samples. One species in particular, Cetobacterium somerae, dominated the Nile tilapia gut microbiome representing 76% of the total sequences. Barnesiella was the second most abundant genus comprising 6.86% of all sequences.

When the gut microbiome composition was compared between different diet treatments, no significant differences were observed. ANOSIM analysis of OTU data for all samples indicated high similarity between all diets with a global R value of -0.022 at a significance level of 55.4% (p=0.554). Therefore, diet treatments did not significantly alter the tilapia gut microbiome. The effect of the different diets on the gut microbiome, if any, was outweighed by the variability between replicates within each treatment (tank effect). The variability observed among replicates is displayed in **Figure 4**. The lack of clarity in clustering was supported by statistical analysis. Likewise, SIMPER analysis of bacterial phyla and genera showed high similarity among the diet treatments **(Table 4)**. At the phylum level, the main difference between diets was due to varying abundances of Fusobacteria and Bacteroidetes. At the genus level, most of the differences between groups were associated with varying abundances of Cetobacterium and Barnsiella. Parabacteroides also contributed to variations between diet treatments but to a lesser extent than those previously mentioned.

Discussion

Nile tilapia is the most commonly cultured tilapia species worldwide and one of the most extensively studied aquaculture species (FAO, 2010). Recently, several studies have looked at what factors shape and modulate the development of the tilapia gut microbiome (Giatsis et al., 2015, Giatsis et al., 2014). During early ontogenetic stages, while the gut is not fully developed, the effects of environmental factors such as water physicochemical parameters are stronger. Those studies concluded that water in the rearing tanks is the primary source for bacteria found in the tilapia gut. However, little information is available on how the mature gut microbiota in tilapia responds to external factors including the use of prebiotics and feed supplements.
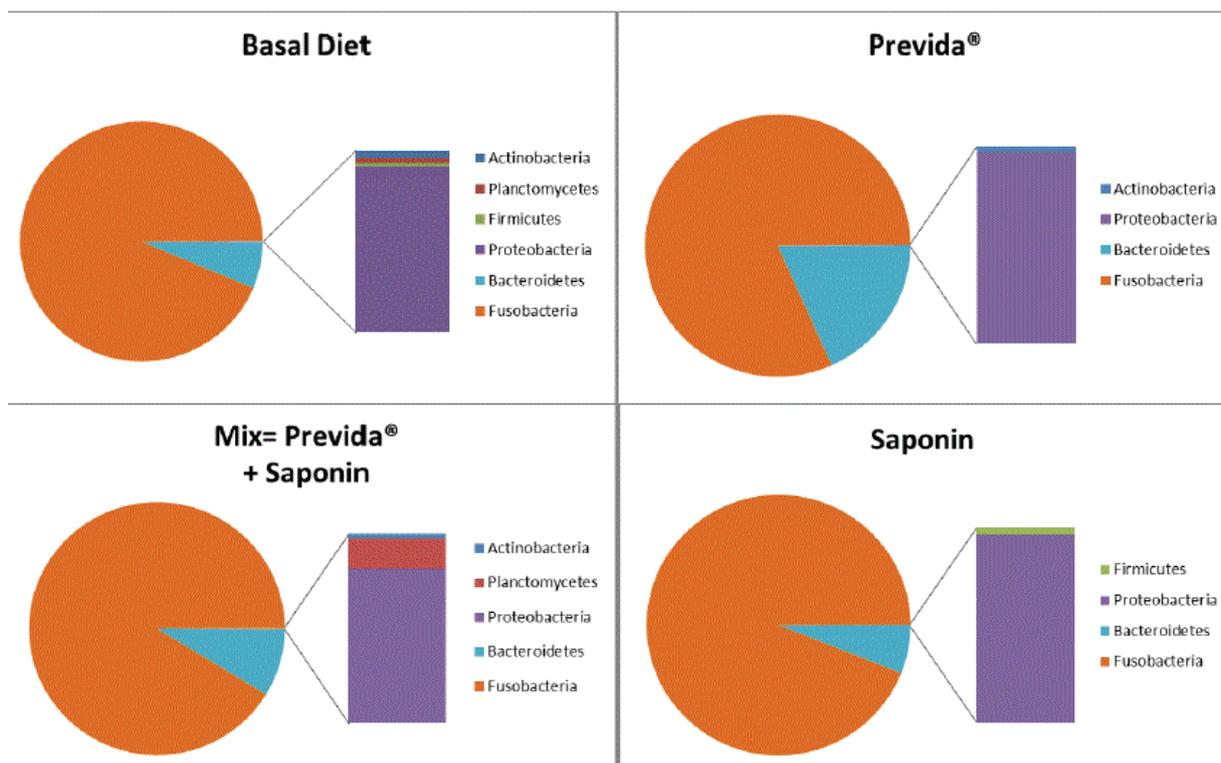
Figure 3: Phylum composition of each diet as obtained through sequencing.

The results of this study showed that the gut microbiota of our experimental Nile tilapia was dominated by the phylum Fusobacteria, specifically by the genus Cetobacterium. In the control diet, more than 90% of the OTUs were ascribed to this genus. This result is in agreement with previous data from omnivorous warmwater fish species collected from the wild (Larsen et al., 2014b). Although percent of abundance was not specified, the study by Ran et al. (2015) also showed Firmicutes as one of the predominant phyla in Nile tilapia reared in aquaculture facilities (Ran et al., 2015). However, other studies have shown that Firmicutes account for >90% of the gut microbiota in Nile tilapia (Standen et al., 2015). Discrepancies in microbiota composition can be due to how the animals were raised and fed, the analysis of autochthonous versus allochthonous bacterial communities, the methodology used for DNA extraction, and sequencing platform and pipeline data analysis (Ringo et al., 2015, Larsen et al., 2014b).

Diets amended with Previda®, saponin, and a combination of the two resulted in no significant changes to the gut microbiome or growth rates of Nile tilapia after two weeks of dietary conditioning. This was unexpected in the case of saponin as the fecal pellets exhibited a much softer consistency than those from other diets. The overwhelming predominance of the genus Cetobacterium and the observed variability among replicates probably masked any subtle effect of the diets in the gut microbiome. Clearly, Cetobacterium was not significantly affected by any of the feed additives. The only observed trend was on diets containing Previda® that did appear to show an increase in populations of Barnesiella with corresponding decreases in the numbers of Cetobacterium. However, only two of the five replicates had a significant increase of Barnesiella numbers. If this trend is correct, it may suggest the treatment regimens and/or durations may not have been adequate to allow for significant changes to occur.

Table 3: Genus identity of sequences represented by percentage from the total sequences obtained for each diet.

Classification				Abundance % (within diets)			
Phylum	Class	Family	Genus	Basal	Previda	S+P mix	Saponin
		Shared Groups					
Fusobacteria	Fusobacteria	Fusobacteriaceae	*Cetobacterium*	93.57%	81.63%	91.21%	93.94%
Bacteroidetes	Bacteroidia	Porphyromonadaceae	*Parabacteroides*	3.00%	3.93%	2.56%	1.91%
Bacteroidetes	Bacteroidetes	Porphyromonadaceae	*Barnesiella*	3.23%	14.32%	6.05%	3.99%
Proteobacteria	γ-Proteobacteria	Enterobacteriaceae	*Plesiomonas*	0.08%	0.08%	0.09%	0.05%
Proteobacteria	γ-Proteobacteria	Pseudomonadaceae	*Pseudomonas*	0.03%	0.01%	0.02%	0.06%
		Unique Groups					
Fusobacteria	Fusobacteria	Fusobacteriaceae	*Fusobacterium*	0.00%	0.01%	0.00%	0.00%
Bacteroidetes	Flavobacteria	Flavobacteriaceae	*Cloacibacterium*	0.00%	0.01%	0.00%	0.00%
Bacteroidetes	Flavobacteria	Flavobacteriaceae	*Marixanthomonas*	0.00%	0.00%	0.00%	0.01%
Bacteroidetes	Flavobacteria	Cryomorphaceae	*Owenweeksia*	0.00%	0.00%	0.00%	0.01%
Bacteroidetes	Sphingobacteria	Chitinophagaceae	*Niabella*	0.01%	0.00%	0.00%	0.00%
Proteobacteria	α-Proteobacteria	Rickettsiales	*Candidatus Captivus*	0.00%	0.00%	0.00%	0.01%
Proteobacteria	α-Proteobacteria	Sphingomonadaceae	*Novosphingobium*	0.03%	0.00%	0.00%	0.00%
Proteobacteria	α-Proteobacteria	Rickettsiaceae	*Orientia*	0.00%	0.00%	0.01%	0.00%
Proteobacteria	β-Proteobacteria	Comamonadaceae	*Acidovorax*	0.01%	0.00%	0.00%	0.00%
Proteobacteria	β-Proteobacteria	Neisseriaceae	*Deefgea*	0.00%	0.00%	0.00%	0.00%
Proteobacteria	β-Proteobacteria	Burkholderiaceae	*Polynucleobacter*	0.00%	0.00%	0.03%	0.00%
Proteobacteria	γ-Proteobacteria	Moraxellaceae	*Acinetobacter*	0.01%	0.00%	0.00%	0.00%
Proteobacteria	γ-Proteobacteria	Xanthomonadaceae	*Arenimonas*	0.00%	0.00%	0.00%	0.00%
Proteobacteria	γ-Proteobacteria	Enterobacteriaceae	*Klebsiella*	0.02%	0.00%	0.00%	0.01%
Actinobacteria	Actinobacteria	Frankiales	*Frankia*	0.01%	0.00%	0.00%	0.00%
Actinobacteria	Actinobacteria	Micrococcaceae	*Micrococcus*	0.00%	0.00%	0.00%	0.00%
Actinobacteria	Actinobacteria	Mycobacteriaceae	*Mycobacterium*	0.00%	0.00%	0.00%	0.00%
Firmicutes	Bacilli	Staphylococcaceae	*Jeotgalicoccus*	0.00%	0.00%	0.00%	0.01%
Firmicutes	Clostridia	Clostridiaceae	*Sarcina*	0.00%	0.00%	0.00%	0.00%
Planctomycetes	Planctomycetacia	Planctomycetaceae	*Rhodopirellula*	0.01%	0.00%	0.00%	0.00%
Planctomycetes	Planctomycetacia	Planctomycetaceae	*Singulisphaera*	0.00%	0.00%	0.03%	0.00%
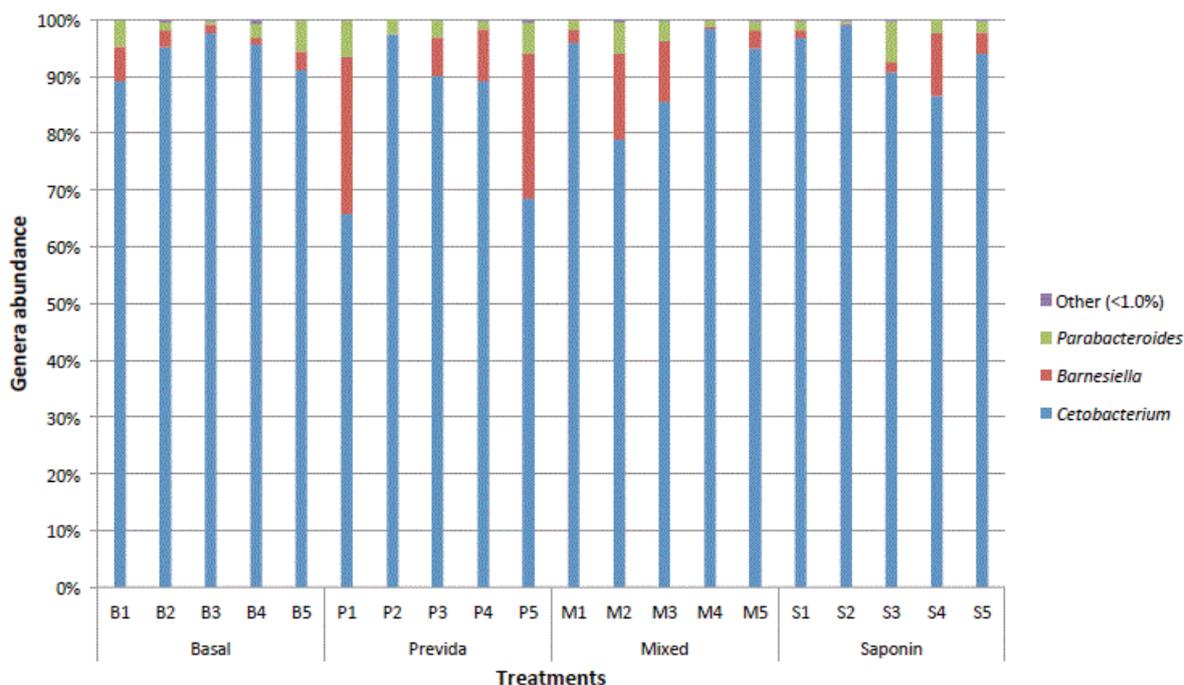
Figure 4: Distribution of predominant genera in each diet obtained by pyrosequencing. Results from each individual replicate are shown.

Table 4: Sequences contributing to the dissimilarity observed between the bacterial communities in pairwise comparisons (B, basal diet; P, Previda®; M, mixed diet; S, saponin).

Groups (I and II)	Overall average dissimilarity	Bacterial Genus	Group I average abundance	Group II average abundance	Contribution to Dissimilarity (%)
B and P	13.85	*Cetobacterium*	15051	11540	47.33
		Barnesiella	519	2024	43
B and M	7.03	*Cetobacterium*	15051	12434	47.2
		Barnesiella	519	824	35.79
		Parabacteroides	482	349	14.34
P and M	13.84	*Cetobacterium*	11540	12434	49.32
		Barnesiella	2024	824	41.73
B and S	5.19	*Cetobacterium*	15051	11663	43.04
		Barnesiella	519	495	30.44
		Parabacteroides	482	237	23.04
P and S	14.2	*Cetobacterium*	11540	11663	47.35
		Barnesiella	2024	495	42.59
		Parabacteroides	556	237	9.12
M and S	7.59	*Cetobacterium*	12434	11663	47.1
		Barnesiella	824	495	36.32
		Parabacteroides	349	237	14.48

Several studies have examined the effects of Previda® on growth, immune response, and the gut microbiome of various aquatic species with mixed results (Burr et al., 2012, Anuta et al., 2014, Zhou et al., 2010). As with the present study, the work by Anuta et al. (2014) found no significant differences in growth rates, weight gain, or survival of test organisms, but gut communities between control and Previda® appeared different (microbial community data were not statistically analyzed). Zhou et al. (2010) found that red drum, Sciaenops ocellatus, fed with Previda® had significantly improved weight gain and

immunological response when compared to basal diets and other commercial prebiotics, but the composition of the gut microbiome was not analyzed in the study.

The high abundance of C. somerae in our experimental tilapia, as well as other previously analyzed freshwater fishes (Larsen et al., 2014a, Li et al., 2015), fuels questions about the functional relationship between this bacterium and its host. C. somerae was originally isolated from the feces of children with late-onset autism that were being treated with vancomycin (Finegold et al., 2003, Tsuchiya et al., 2008). Strains have been found to be capable of producing B12 at relatively high rates across different fish species (Sugita et al., 1991). Studies have suggested fishes with predominant populations of some obligate anaerobes, such as C. somerae, are less likely to suffer from signs of B12 deficiency when fed diets lacking B12. Based on their collections, Sugita et al. (1991) reported that C. somerae could be isolated from carp (*Cyprinus carpio*), tilapia, goldfish (*Carassius auratus*), and ayu (*Plecoglossus altivelis*), but not from Japanese eel (*Anguilla japonica*) or channel catfish (*Ictalurus punctatus*). C. somerae was found most frequently in tilapia and carp, which have been shown to not require B12 supplementation in diets suggesting that C. somerae plays an important role in B12 production (Sugita et al., 1991).

Barnesiella was the second most common genus found in samples comprising 6.87% of all sequences. To our knowledge, this is the first known report of this genus in fishes. In humans and mice, Barnesiella has been demonstrated to show promise in the reduction or elimination of vancomycin-resistant Enteroccocus faecium (Ubeda et al., 2013), but little is known about other functional roles that this genus might play in the host gastrointestinal tract.

The present study showed a relatively low diversity of the gut microbiome in our experimental Nile tilapia. This can be due to several factors including genetic lineage, rearing conditions, and feeding regimes. It is noteworthy that a low diversity translated into a resilient community that did not significantly change when modified diets were applied. It is plausible that the duration of the study might have been too short to allow for significant bacterial change to occur. Another explanation is that once the gut matures and the gut communities get established they become harder to modify than those during the early stages of development. Future studies should evaluate the use of prebiotics at different ontogenetic stages to determine the best window to exert the maximum effect on the gut communities while they are being formed.

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