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Impact of *Arthrospira maxima* Feed Supplementation on Gut Microbiota and Growth Performance of Tilapia Fry (*Oreochromis niloticus*)

Dorian Rojas-Villalta [1], Olman Gómez-Espinoza [2,3,*], Rossy Guillén-Watson [1,3], Francinie Murillo-Vega [1], Fabián Villalta-Romero [1], Felipe Vaquerano-Pineda [4], Mauricio Chicas [1], Maritza Guerrero [1] and Kattia Núñez-Montero [5,*]

[1] Biotechnology Research Center, Department of Biology, Instituto Tecnológico de Costa Rica, Cartago 159-7050, Costa Rica; rojasvillaltadorian@gmail.com (D.R.-V.); roguillen@itcr.ac.cr (R.G.-W.); frmurillo@itcr.ac.cr (F.M.-V.); fvillalta@itcr.ac.cr (F.V.-R.); mchicas@itcr.ac.cr (M.C.); mguerrero@itcr.ac.cr (M.G.)
[2] Departamento de Ciencias Químicas y Recursos Naturales, Facultad de Ingeniería y Ciencias, Universidad de La Frontera, Temuco 4811230, Chile
[3] Facultad de Ingeniería, Universidad Autónoma de Chile, Temuco 4810101, Chile; rossy.guillen@cloud.uautonoma.cl
[4] Escuela de Agronegocios, Instituto Tecnológico de Costa Rica, Cartago 159-7050, Costa Rica; fvaquerano@itcr.ac.cr
[5] Facultad Ciencias de la Salud, Instituto de Ciencias Aplicadas, Universidad Autónoma de Chile, Temuco 4810101, Chile
* Correspondence: olman.gomez@ufrontera.cl (O.G.-E.); kattia.nunez@uautonoma.cl (K.N.-M.)

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Abstract: Microalgae are promising as prebiotics in aquaculture. *Arthrospira maxima* has potential nutritional value but is poorly studied. We assessed *A. maxima* feed supplementation in tilapia fry and evaluated its effect on growth performance and gut microbiota. Fish were cultivated in ponds under 0%, 5%, 10%, and 15% *A. maxima* inclusion treatments. Growth parameters and biomass proximate analysis were assessed. A meta-amplicon analysis was performed on the gut microbiota using DADA2 and PICRUST2 for functional prediction. Among treatments, the 5% supplementation group seemed to present no negative effect on growth parameters and did not compromise the nutritional quality of tilapia fry biomass. Microbial composition was characterized by *Cetobacterium*, *Pseudomonas* and *Aeromonas* genera, and a predominance of beneficial metabolic pathways. Microbiota of tilapia fry fed with *A. maxima* showed unique genera with reported beneficial functions in tilapia. The abundance of potential pathogenic taxa was significantly decreased in supplementation treatments, possibly related to valuable compounds of *A. maxima*. The inclusion of the microalgae supported the stability of the microbiota, favoring the growth of commensal species. This seems to have an effect on decreasing the presence of pathogenic genera in the gastrointestinal tract. Overall, our work proposes 5% feed inclusion of *A. maxima* to be the most suitable for tilapia fry aquaculture to maintain production rates while improving general health.

Keywords: aquaculture; microalgae supplementation; gastrointestinal microbiota; meta-amplicon analysis; microbiome profiling

Key Contribution: We provide insights into the utilization of *A. maxima* as a feed supplement for tilapia fry aquaculture.

1. Introduction

The fish commonly referred to as 'tilapia' includes species within the family Cichlidae. These organisms are one of the main animal protein sources for human consumption, ranking as the second most produced in fish farming globally [1]. In recent years, global

production of tilapia has been estimated at up to 6.8 million tons in 2020 [2]. Statistics show that only 0.7 million tons (approximately 10%) are obtained by capture, while the remaining 90% is produced by aquaculture farms [2]. The rise in the global population has increased the demand for food worldwide, demanding an expansion in production yields.

Aquaculture of fish facilities better yields and utilization of resources [3]. In this sense, aquaculture of tilapia has benefited the fishery industry by more than doubling the production compared to 20 years ago [2]. Despite its advantages, several challenges have emerged over the years, including microbial diseases in fish. To prevent illness, farmers have resorted to the use of vaccines, antibiotics and parasiticides. However, their cost represents a significant financial burden, and in some cases, their utilization is constrained [4]. Therefore, cheaper yet effective alternatives to vaccines and antibiotics are a hot spot for research. Among these, supplementation with prebiotics, probiotics, and other feed supplements has been explored [5]. Prebiotics are non-digestible compounds, and probiotics are live microbial supplements. Both pre- and probiotics benefit health status by stimulating the gastrointestinal (GI) microbial balance towards the growth of beneficial bacteria and combating undesirable microorganisms [5]. Among these alternatives, microalgae emerge as a promising solution.

Microalgae is a general term for photosynthetic microorganisms from prokaryotic (cyanobacteria) and eukaryotic (green, red, and other microalgae) taxonomic domains. Recently, the biotechnological application of microalgae has been widely studied. These microorganisms are known to present a unique nutritional profile with balanced percentages of carbohydrates, protein, lipids, vitamins, and minerals [6]. Additionally, microalgae can produce bioactive compounds with antioxidant, antimicrobial, antiparasitic and antiviral properties [7]. These characteristics position microalgae as promising feed supplements, functioning as pre- and probiotics [7,8].

It has been proposed that microalgae-based supplements promote growth of tilapia fry and protect against diseases by beneficially affecting the GI microbial communities [7,9]. Previous studies have supported this idea by showing that feeding fish in aquaculture with microalgae supplements results in bigger, healthier, and disease-free fish [10,11]. Also, meta-amplicon studies of the GI microbiota of tilapia fed with microalgae demonstrated an increase in the relative abundance of beneficial bacteria [12].

Arthrospira (also known as spirulina) species are relevant edible microalgae due to their high nutritional value [13]. Among them, *A. platensis* has been extensively studied as a feed supplement [14]. Previous research in tilapia aquaculture systems has reported that the inclusion of *A. platensis* at percentages of 30% increases growth, feed utilization efficiency, and improves the health status of fish [15]. Other authors have reported enhanced activity of antioxidant enzymes (e.g., superoxide dismutase, catalase) in tilapia individuals fed with *A. platensis* supplemented meal [16].

While *A. platensis* is widely researched in this regard, other promising species, such as *A. maxima*, are poorly explored. Biochemical characterization of *A. maxima* revealed total protein and lipid contents of ~43% and ~4%, respectively, along with high antioxidant capacity [17]. These values are similar to or slightly higher than those reported for *A. platensis*, indicating its potential as a feed supplement. Additionally, previous studies have shown that microalgae might enhance the growth performance of tilapia [18]. These characteristics support the idea that it is a suitable microorganism to increase tilapia production yields.

Therefore, studying the growth performance and bacterial dynamics of tilapia supplemented with microalgae in aquaculture is relevant, as it might provide insights into optimal growth conditions to maximize production yields. Hence, in this research, we aimed to assess the implementation of different concentrations of *Arthrospira maxima* (spirulina) as a feed supplement in tilapia fry (*Oreochromis niloticus*) and evaluate its effect on growth performance and GI microbiota.

2. Materials and Methods

2.1. Arthrospira Maxima Culture and Biomass Harvesting

Microalgae biomass was produced at the Bioenergy Laboratory of Biotechnology Research Center (CIB, Instituto Tecnológico de Costa Rica). Culture medium was based on an adapted industrial formulation of Zarrouk, supplemented with 10 g/L of $NaHCO_3$ as carbon source and pH regulator (range between 9 and 10). The initial inoculum for culture was 0.1 g/L (1.7×10^5 cells/mL). Chemical conditions were monitored according to Poveda-Víquez et al. (2023), and a similar production system and conditions were also used as described [19]. Briefly, a raceway system (water level of 0.4 m deep) was employed to maintain the culture in a continuous system and a volumetric capacity of 30,000 L. Cultivation lasted 35 days, and cell growth was evaluated by optical microscope (DM 750, Leica Microsystem, Wetzlar, Germany) and a Neubauer cell counting chamber. Dry weight was monitored through a halogen thermobalance (Radwag Balances and Scales, Toruńska, Poland). Biomass density and culture yield reached an optimal level of 0.71 g/L and 0.98 g/L, respectively, on day 35. Biomass was harvested weekly, centrifugation (4200 rpm, SSD 606,007 Gea Westfalia separator, GEA, Düsseldorf, Germany), and spray-dried using the Galaxie ECO Dryer® 1512 (Galaxie, Buenos Aires, Argentina). The final product was a fine green powder of the *A. maxima* biomass with a particle size of 150 µm.

2.2. Microalga-Based Feed Preparation

For feeding trials, the commercial Tilapia feed, Acuaoro Tilapia (Belina, Cartago, Costa Rica), served as the control diet (particle size: <1000 µm, 0 × 0). We performed a physical incorporation of the microalgae (experimental diets). Briefly, in a sterile recipient, commercial feed was mixed with the corresponding percentage of *A. maxima* biomass (5%, 10%, or 15%). The binder Pegalaq (Laquinsa Salud Animal, Cartago, Costa Rica) was later added at a concentration of 75 mL per kilogram of feed and all the ingredients were thoroughly stirred. This binder is used to ensure the correct inclusion of microalgae biomass to the commercial feed, avoiding its later dissociation when thrown to bioassay tanks.

To assess the nutritional quality of both the commercial feed and the *A. maxima* biomass, proximate analyses were performed at Asesorías Químicas Y Laboratorio A.Q.Y.L. S.A., Costa Rica. These analyses evaluated moisture, crude protein, crude fat, ash, and fiber content in accordance with the AOAC Methods of Analysis 2010. The nutritional parameters of experimental diets were estimated by addition of the corresponding percentages based on values from proximate analysis (Table 1).

Table 1. Nutritional parameters from proximal analysis of commercial feed, pure *A. maxima* biomass, and estimation of experimental diets.

Parameter (% m/m)	Acuaoro Tilapia Feed	*A. maxima* Biomass	5% Supplementation [a]	10% Supplementation [a]	15% Supplementation [a]
Moisture	9.63 ± 0.01	17.1 ± 0.2	10.00 ± 0.01	10.38 ± 0.03	10.75 ± 0.04
Ash	9.90 ± 0.04	9.66 ± 0.04	9.89 ± 0.04	9.88 ± 0.04	9.86 ± 0.04
Crude fiber	2.38 ± 0.07	<0.1	2.27 ± 0.07	2.15 ± 0.07	2.04 ± 0.07
Protein	36.8 ± 0.1	50.97 ± 0.03	37.51 ± 0.1	38.22 ± 0.09	38.93 ± 0.09
Total fat	9.1 ± 0.3	5.06 ± 0.04	8.90 ± 0.29	8.70 ± 0.27	8.49 ± 0.26
Ether extract	7.4 ± 0.4	5.0 ± 0.1	7.28 ± 0.39	7.16 ± 0.37	7.04 ± 0.36
Carbohydrates	33.9 ± 0.6	17.2 ± 0.4	33.07 ± 0.59	32.23 ± 0.58	31.40 ± 0.57

[a] Values from experimental diets were calculated by adding the corresponding percentage based on the values from proximate analyses of pure commercial feed and *A. maxima* biomass.

2.3. Bioassay Fish Selection

A total of 1200 newly hatched male monosex tilapia *Oreochromis niloticus* (initial weight: 0.02 g) were utilized for this study (Biopez, Costa Rica). The feeding trial began 9 days post-hatch, following a 9-day acclimation period on commercial feed to adapt the fish to their new environment. The quantity of feed administered was determined in rates of

2% to 3% of body weight (BW), based on manufacturers guidelines. The bioassay was designed as a simple randomized study. Each experimental unit consisted of 50 gallons 8 mm fiberglass tanks with controlled conditions to promote optimal tilapia growth. The tanks were installed with two activated carbon filters (DoPhin F1200 internal filter, Cartago, Costa Rica), an air pump (Xilong XL-2187, Cartago, Costa Rica), white LED light (AROWAN 40 cm lamp, Cartago, Costa Rica), a thermostat (Xilong 200 W, Cartago, Costa Rica), and a glass thermometer (Aquadene, Cartago, Costa Rica). The bottom of the experimental units was decorated with 10 cm of fine river rocks. For instance, temperature maintained at 29–32 °C, dissolved oxygen (maintained at levels above 6 mg/L), pH (7.5 ± 0.5), and ammonia nitrogen (<0.04 mg/L), were monitored daily to ensure optimal living conditions.

2.4. Experimental Design

The bioassay was carried out at the Laboratorio de Sanidad Animal, which is part of the Escuela de Agronegocios at the Instituto Tecnológico de Costa Rica (ITCR), situated in the Cartago province. The experiment was structured into four distinct dietary treatment groups fed with the baseline diet supplemented with the addition of 0% (control), 5%, 10% and 15% microalgae biomass. The treatments were named C, A, B, and D, respectively. Each of the four treatments presented three replicates, with 100 fish allocated per replicate, totaling 12 experimental units. Bioassay was conducted over a period of 8 weeks to assess the effects of *A. maxima* supplementation on tilapia growth performance and gut microbiota dynamic.

2.5. Bioassay Monitoring

Weekly measurements of ammonia were also performed throughout the 56-day bioassay period. Feeding was conducted manually twice daily, in the morning (8:00–9:00) and in the evening (17:00–18:00), at rates of 2% to 3% BW per feeding, following the feeding guidelines provided by the commercial feed manufacturer. This regimen was adjusted weekly based on growth to ensure the provision of an isocaloric diet and to prevent feeding stress.

For biomass weekly assessment, ten fish from each tank were randomly sampled, measuring length, width, weight, and head size. Growth performance was evaluated through weekly weight measurements, and the data was analyzed using the allometric equation Y = a × b to correlate growth parameters. Calculations of the final biomass and Feed Conversation Ratio (*FCR*) were accessed as follows. First, the *final biomass*:

$$Final\ biomass = number\ of\ fish \times average\ weight\ (g) \quad (1)$$

where the '*number of fish*' is the quantity alive at the end of the experiment, and the *FCR*:

$$FCR = \frac{final\ biomass\ (g)}{Total\ used\ feed\ (g)} \quad (2)$$

The Specific Growth Rate (*SGR*) was calculated using the following formula:

$$SGR = \frac{\ln(final\ weight) - \ln(initial\ weight)}{bioassay\ duration\ (days)} \times 100 \quad (3)$$

where '*final*' and '*initial weight*' was given in grams. Additionally, a proximal analysis of the final biomass of the fry from each treatment was carried out at the end of the experiment in accordance with the AOAC Methods of Analysis 2010. A total harvest and count of the fish were conducted to determine *survival* rates as follow:

$$Survival\ (\%) = \frac{fish\ alive}{total\ fish} \times 100$$

where '*fish alive*' represents the fish alive at the end of the bioassay and '*total fish*' is the number of individuals per fiberglass tank at the beginning of the experiment (100 fish).

2.6. DNA Extraction, 16S rRNA Gene High-Throughput Sequencing, and Meta-Amplicon Analysis of Gut Microbiota

2.6.1. DNA Extraction and 16S rRNA Gene Amplicon Sequencing

The intestines of three fish per treatment were obtained with a sterilized dissecting knife, immediately frozen in liquid nitrogen, and stored in a −80 °C freezer. The total DNA of *O. niloticus* complete gut (including its content) was extracted through the DNeasy® PowerSoil® Pro kit following manufacturer's instructions with modifications: (i) a complete tilapia gut was mixed with CD1 solution and incubated at 60 °C for 10 min in a thermoblock (AccuBlock™ Digital Dry Bath, Labnet International, Edison, NJ, USA). (ii) After incubation, samples were lysed using a mechanical macerator in cycles of 5 s at 15 s^{-1} until it was partially homogenized. (iii) Prior dilution, column with CD6 buffer was let to rest for 2 min and later centrifuged. Extracted DNA was stored in a −80 °C freezer until further use.

The quality and purity (260/280 and 260/230 absorption ratios) of extracted DNA was assessed using the QuatiFlour® ONE dsDNA System kit on a Quantus device (Promega, Madison, WI, USA), and Nanodrop Lite Spectrophotometer (Thermo Scientific, Waltham, MA, USA), respectively. The integrity of the samples was verified with 1% agarose gel. Subsequently, the obtained DNA was sent for amplification and sequencing to Macrogen, Inc. (Seoul, Republic of Korea).

2.6.2. Meta-Amplicon Analysis of Gut microbiota

The genomic library was prepared with the Herculase II Fusion DNA Polymerase Nextera XT Index V2 kit. The hypervariable V3–V4 region in the 16S rRNA gene was sequenced through a 2 × 300 bp technique on an Illumina Miseq platform (Seoul, Republic of Korea). For this, the primers Bakt_341F (5′-CCTACGGGNGGCWGCAG-3′) and Bakt_805R (5′-GACTACHVGGGTATCTAATCC-3′) were used.

For meta-amplicon analysis, a total of 24 raw reads files were received, corresponding to forward and reverse files of each of the three replicates from 4 treatments. The DADA2 R package v1.30.0 [20] was used to perform the analysis [21]. First, the raw reads quality was examined using 'plotQualityProfile()' function. Later, sequences were trimmed with 'filterAndTrim()' command (maxEE = 2, and truncLen values of 300 and 270 for forward and reverse data, respectively). Sequence errors were estimated with the 'learnError()' model. The 'dada()' algorithm for error correction and denoising was applied. The corrected sequences were merged using 'mergePairs()' function, resulting in 12 .fastq files. An amplicon sequence variants (ASVs) count table was created by 'makeSequenceTable()' method. The chimeric sequences of the table were removed by 'removeBimeraDenovo()' mode of DADA2. A summary of raw sequences during preprocessing can be found in Supplementary Table S1.

For taxonomy assignment, we aligned curated ASVs table against the SILVA rRNA database v138.1 (dataset: silva_nr99_v138.1_train_set.fa) [22]. Alignment was performed through 'assignTaxonomy()' command. ASVs count table, taxonomy table and metadata (.tsv file) were used to create a phyloseq-class object with 'phyloseq()' command from phyloseq v1.46.0 R package [23]. Non-bacterial (archaea, chloroplast, mitochondria), unclassified and taxa with read count less than 5 were removed using 'tax_fix()' function from microViz v0.12.1 R package [24]. The phyloseq-class object was converted into a data frame by 'psmelt()' option. From the data frame, unique, shared, and potential pathogenic genera (according to [25]) were retrieved. Graphical data visualization was generated through ggplot2 R package v3.5.0 [26].

ASVs sequences and abundance were extracted from the DADA2 object through script and used to run the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUST2) v2.5.2 [27]. PICRUST2 aligns ASVs sequences to reference species tree and associates to KEGG gene family copy number per reference genome [27]. Later, the abundance table of ASVs is combined to the related genes abundance to determine the relative abundance per sample. Finally, genes profiles are mapped into gene pathways to output the predicted metabolic pathways relative abundances [27]. Data was visualized

through a heatmap with 'pheatmap()' function from pheatmap v1.0.12 R package [28]. Euclidean distances were calculated using the factoextra R package v1.0.7. Except otherwise noted, all commands were used with default parameters.

2.7. Statistical Analysis

Growth parameters and proximal composition were accessed using Shapiro–Wilk and Levene test. Results showed that all data followed a normal distribution and could be considered parametric ($p > 0.05$), except for moisture ($p < 0.05$). To determine the significant difference among parameters, we performed a One-way analysis of variances (ANOVA) for parametric data. For non-parametric data, statistical difference was calculated through Kruskal–Wallis test. To differentiate samples with a significant difference, Tukey's HSD (Honestly Significant Difference) and post hoc Nemenyi tests were employed for parametric and non-parametric data, respectively.

Regarding meta-amplicon data, rarefaction curves were calculated based on observed features of ASVs count table through the 'rarecurve()' function from vegan R package v2.6.4 [29]. To assess alpha diversity, we used the curated taxonomy to estimate richness (observed and Chao1) and diversity indexes of Shannon and Simpson using the 'estimate_richness()' phyloseq command. Shapiro–Wilk and Levene tests revealed our data followed a normal distribution ($p > 0.05$), except for the Simpson index values ($p < 0.05$). Hence, significant differences among the alpha diversity were evaluated with an ANOVA test and Kruskal–Wallis test, for parametric and non-parametric values, respectively. To evaluate variations among treatments, we calculated Bray–Curtis dissimilarities using microViz, aggregating per 'Genus'. Later, significant differences among values were accessed through a permutational multivariate analysis of variance (PERMANOVA), establishing 1000 permutations. We visualized the Bray-Curtis distances on Principal Coordinate Analysis (PCoA) and non-metric multidimensional scaling (NMDS) analysis with microViz and phyloseq, respectively. Abundances of pathogenic genera and predicted metabolic pathways were analyzed using Shapiro–Wilk and Levene test. All data were categorized as parametric ($p > 0.05$), and consequently accessed using ANOVA and Tukey's HSD. All graphics were generated with ggplot2, except otherwise noted.

3. Results and Discussion

3.1. Growth and Nutrional Parameters of Tilapia Fry Supplemented with Arthrospira Maxima

Our results showed no significant difference in the growth performance parameters ($p > 0.05$) (Table 2). This indicates that microalgae supplementation did not limit or challenge the tilapia fry growth or survival. Additionally, the correlation analysis showed an association between the length and final weight of fish supplemented with 5% of *Arthrospira maxima* ($R^2 = 0.98$). No other treatment or measurement presented a correlation ($R^2 < 0.95$).

Table 2. Parameters evaluated for growth performance of tilapia fry (*Oreochromis niloticus*) fed with different concentrations of microalgae supplement during the 2-month bioassays.

Treatment	Length (cm)	Width (cm)	Final Weight (g)	Survival (%)	Final Biomass (g)	FCR	SGR (%/d)
5%	5.74 ± 0.31	2.22 ± 0.10	7.50 ± 1.18	63.00 ± 7.94	467.13 ± 36.61	1.65 ± 0.21	10.1 ± 0.3
10%	5.30 ± 0.34	2.11 ± 0.21	6.13 ± 1.55	60.33 ± 9.50	361.37 ± 46.33	2.46 ± 0.32	10.4 ± 0.3
15%	5.47 ± 0.26	2.08 ± 0.04	6.96 ± 1.33	58.33 ± 7.64	408.87 ± 106.45	2.71 ± 0.92	10.4 ± 0.2
Control	5.28 ± 0.36	2.11 ± 0.18	6.60 ± 0.94	57.00 ± 17.44	370.07 ± 104.91	2.21 ± 0.87	10.4 ± 0.3

Values represent the average of triplicated measurements and respective standard deviation. FCR = Feed Conversion Ratio; SGR = Specific Growth Rate.

To our knowledge, few studies have tested the performance of *A. maxima* as a feed supplement for tilapia. Hence, our study pioneers the characterization of this microalga's potential application in tilapia aquaculture. One related study demonstrated similar results, where *A. maxima* supplementation had no significant impact on most growth parame-

ters [18]. Only the feed conversion ratio (FCR) differed in the 30% *A. maxima* supplementation treatment (1.87) [18]. Furthermore, diets with this microalga have been studied in other fish species, showing similar results. A study implementing *A. maxima* in the feed of Betta fish (*Betta splendens*) significantly affected growth when using 15% inclusion percentage [30].

Other *Arthrospira* species have been widely studied, especially *A. platensis.* Research has demonstrated a significant impact of *A. platensis* on tilapia growth performance [31]. An experiment testing 30%, 45%, 60% and 75% *A. platensis* supplementation found 30% treatment as the optimal diet [15]. Other treatments had a lower growth performance and decreased fish survival rates [15]. Moreover, FCR values for *A. platensis* supplementation range from 0.84–1.80, with 30% inclusion treatment holding the lowest value [16,32]. For *A. maxima* supplementation trials, a range of 1.87–2.09 FCR is reported [18]. Overall, our results showed FCR varying between 1.65–2.71, values notably above those found in literature. The lowest value (1.65 $p > 0.05$) resulted from 5% supplementation group.

Another important growth parameter is the specific growth ratio (SGR). To our knowledge, SGR has not been previously measured in studies involving *A. maxima* supplementation in tilapia fry aquaculture systems. However, SGR values for fish supplemented with *A. platensis* range from 2.64% to 4.64% [15,32]. In earlier studies, *A. platensis* was used to replace protein in commercial feeds, resulting in higher SGR values of 12.00% to 15.40% in a twice-enhanced feeding trial [16]. In our study, SGR values ranged from 10.1% to 10.4% (Table 2). These percentages are more comparable to those observed in the twice-enhanced feeding trials, rather than the lower values from *A. platensis* supplementation alone. This suggests that *A. maxima* may have a more significant impact on tilapia growth compared to *A. platensis*. However, this hypothesis requires further validation through a bioassay directly comparing the effects of both species in parallel.

The correlation analysis showing a strong association between the length and final weight in the 5% *A. maxima* group indicates that this specific supplementation level might have a positive relationship with growth consistency in tilapia fry, as higher R^2 values suggest a strong linear relationship. However, since no other treatments or measurements showed a correlation ($R^2 < 0.95$), it suggests that the 5% supplementation may be the most effective in promoting a balanced growth pattern, though overall effects may still be limited without significant differences across treatments. The 5% treatment consistently showed favorable trends, including notably high SGR values compared to other studies. Additionally, from a large-scale commercial perspective, achieving significant benefits with a lower amount of algae supplementation can be economically advantageous for producers.

Arthrospira maxima supplementation had a significant impact ($p < 0.05$) on the proximate composition of tilapia fry biomass (except for crude fiber and protein, $p > 0.05$) (Table 3). Protein content reached its highest value under the 10% treatment (14.5% $\pm$ 0.7), although it did not differ significantly from other groups. Total lipid percentage decreased as the percentage of microalgae supplementation increased, with the lowest values observed in the 10% and 15% treatments. This behavior is expected and has been reported by other authors [16,33]. Microalgae contain compounds such as polyphenols, which have fat-reducing properties, naturally lowering the lipid content in fish [33].

Similar studies found close values to those measured in our proximal analysis. With *A. platensis* supplementation, the protein and lipid content ranges from 14.34% to 16.40% (m/m) and 2.17% to 2.86% (m/m), respectively [15]. Interestingly, enhanced nutritional properties of tilapia fry are noticeable on 10–30% inclusion treatments [15,16]. *Arthrospira* is known to improve the digestive enzyme activity of tilapia fry, due to its phycocyanin and beta-carotene content [16]. Additionally, this microalga presents a cell wall composed mainly of peptidoglycan, a soft compound in comparison to cellulose. This characteristic favors a faster cell disruption and nutrient accessibility [32], which might be partially responsible for enhanced nutritional value of tilapia fry biomass.

Table 3. Proximate composition of tilapia fry biomass after *A. maxima* supplemented feed 2-month bioassays.

Parameter (% m/m)	5%	10%	15%	Control
Moisture	$74.8^{ab} \pm 0.2$	$69.8^{a} \pm 0.2$	$76.1^{b} \pm 0.6$	$75.45^{ab} \pm 0.04$
Ash	$3.3^{a} \pm 0.1$	$3.9^{ab} \pm 0.3$	$4.1^{b} \pm 0.1$	$3.1^{a} \pm 0.4$
Crude fiber	<0.1	<0.1	<0.1	<0.1
Protein	13.0 ± 1	14.5 ± 0.7	13.8 ± 0.1	12.9 ± 0.4
Total fat	$6.64^{b} \pm 0.08$	$5.1^{a} \pm 0.4$	$5.2^{a} \pm 0.09$	$6.5^{b} \pm 0.5$
Ether extract	$6.0^{c} \pm 0.2$	$3.9^{a} \pm 0.1$	$5.1^{b} \pm 0.1$	$5.34^{b} \pm 0.31$
Carbohydrates	$3^{a} \pm 1$	$8^{b} \pm 1$	$<1^{a}$	$3^{a} \pm 1$

Values represent the average of triplicated measurements and respective standard deviation. All parameters presented a significant difference per treatment ($p < 0.05$). Super indexes per row indicate the specific pair of groups differing ($p < 0.05$).

Obtained values for proximal composition was within the range of healthy tilapia fish (protein = 13–25% and total lipids = 0.79–8.5%) [34]. Only carbohydrates showed a higher percentage than reported in literature (carbohydrates < 1%) [34]. The lower supplementation percentage (5%) did not exhibit a significant difference to the control group, avoiding alterations differently to the 15% and 10% percentages. The 15% and 10% treatments evidenced slightly higher values of protein ($p > 0.05$) (Table 3). Excess protein content of tilapia might indicate that fish is required to provide more energy for protein excretions rather than growth. Previous studies showed higher inclusion of crude protein to increase the excretion of nitrogenous compounds, without necessarily benefiting fish growth [35]. This might explain the slightly lower growth parameters values found in these treatments, in comparison to control group and 5% inclusion (Table 2). This statement is also supported by the estimated nutritional composition of experimental diets (Table 1). The energetic macromolecules (fat and carbohydrates) are decreased by the microalgae addition, while protein content increases. We consider higher inclusion percentages (10% and 15%) of *A. maxima* could be compromising the growth as less energetic sources are provided and the diets might have excess protein.

The 5% *A. maxima* supplementation treatment presented the most convenient values among the parameters: a similar protein and carbohydrates percentages to control group. Although a lipid decrease was expected by the addition of the microalgae, *Arthrospira* is part of the natural diet of *Nile tilapia*, as it is common in their natural environment [15]. Some studies have shown that *Arthrospira* can represent up to 25% of the organic matter consumed by wild tilapia [15]. We considered 5% might be a close value to the natural occurring in the control ponds, explaining the similarity to the control group.

These results indicate that 5% inclusion maintains the nutritional value of tilapia fry biomass. Therefore, considering the growth performance, proximate composition, and commercial feasibility, we suggest that 5% *A. maxima* supplementation is a promising candidate for further testing in scaling-up trials. This recommendation is similar to previously reported for *A. platensis* where lower inclusion percentage represented the ideal condition in tilapia and other fish species [16,36]. However, it is different from the one proposed for *A. maxima* (30% inclusion) [18].

Our study marks a new perspective in the potential of *A. maxima* as a feed supplement. We believe a slight supplementation of *A. maxima* favors the maintenance of nutritional quality biomass while improving growth rate in comparison to *A. platensis.* To dive deeper into the understanding of the microalgae inclusion effect on tilapia fry individuals, we evaluated the gut microbiota through a meta-amplicon approach.

3.2. Gastrointestinal Microbiota Community Composition of Tilapia Fry

The 12 tilapia fry gut samples were sequenced and a total of 1,444,700 sequences were obtained. After preprocessing, sequences were lowered to 857,317 reads, with an average of 71,443 ± 18,490 per sample. This represented a total number of 2970 amplicon sequence

variants (ASVs). Rarefaction curves per sample revealed an asymptotic level before the 25,000 reads mark (Supplementary Figure S1). This suggests that estimated richness of ASVs was achieved completely in all samples.

After ensuring samples full estimated richness, taxonomy was assigned to detected ASVs. The gut microbiota of tilapia fry were dominated by two phyla: Fusobacteria (0.27–0.89 of relative abundance) and Proteobacteria (0.07–0.75) (Supplementary Figure S2). Fusobacteria was the most abundant phyla, with Proteobacteria overcoming in only two samples (5% supplementation, second replica, and 15% supplementation, second replica). Interestingly, other phyla presented a higher abundance when Proteobacteria was dominant. Among these, Actinobacteriota and Firmicutes achieve abundances ranging from 0.09–0.15 and 0.03–0.05, respectively.

Our results are consistent with those reported in literature. Fusobacteria, Proteobacteria, Actinobacteria, Planctomycetota, and Firmicutes phyla are commonly found in freshwater species [37]. Microbial co-occurrence networks of cichlids from America and Africa demonstrated major abundance of these phyla across samples [38]. These results provided the idea that these taxa are part of a core microbiota of Cichlidae family [38]. As expected, microbiota study of healthy wild and aquaculture tilapia fish showed the same gut microbial pattern [39–41]. However, regardless consistency, studies demonstrated an dysbiotic microbiota in diseased and fish exposed to contamination, particularly at genus level [39,40].

In the genera composition (Figure 1), *Cetobacterium* was the dominant genus in all samples (0.27–0.89), except for the second replica of 5% treatment (0.08). In this specific sample, *Pseudomonas* represented the most abundant genus (0.53). This result remains unsurprising as *Cetobacterium* is part of the Fusobacteria phylum, while *Pseudomonas* is a Proteobacteria. Both of these were the most abundant phyla in microbiota (Supplementary Figure S2). Moreover, other noticeable genera are *Aeromonas* (0–0.15, Proteobacteria), *Ralstonia* (0.001–0.15, Proteobacteria), *Mycobacterium* (0–0.05, Actinobacteriota), an unclassified genus from the Aeromonadaceae family (0–0.05, Proteobacteria), and *Gemmobacter* (0–0.02, Proteobacteria).

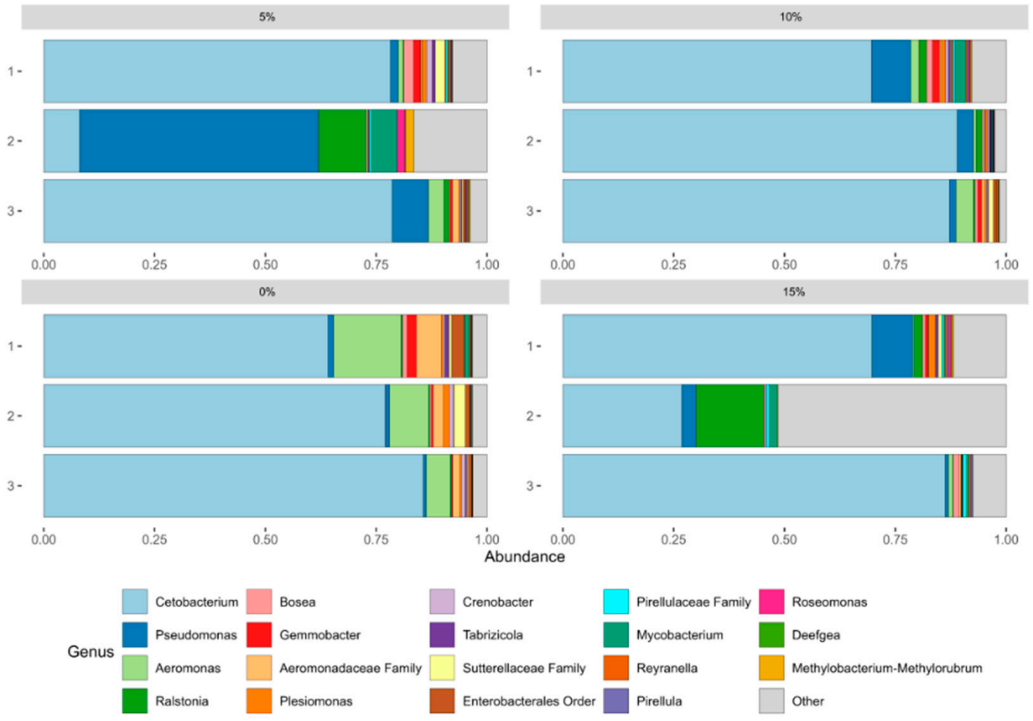

Figure 1. Relative abundance of top 20 most abundant genera in the gut microbiota of tilapia fry fed with *A. maxima* supplemented feed. Associated number refers to the replica.

Cetobacterium, *Aeromonas*, *Mycobacterium*, and *Gemmobacter* are considered part of the core microbiota of tilapia fish [42–44]. Remarkably, *Cetobacterium* is thought to play a key role in tilapia gut. Although the exact function is unclear, its potential for macromolecules biosynthesis insights into a nutrition-related role [45–47]. *Aeromonas* is common in freshwater fish, however, some species represent opportunistic pathogens in tilapia [25]. Moreover, literature is consistent with the presence of *Pseudomonas* in microbiota of tilapia, in lesser abundance [48]. Interestingly, *Ralstonia* presented a higher abundance in 10% and 15% inclusion samples. Some studies have proposed that this genus is related to the cycling of nitrogen [49]. As mentioned above, 10% and 15% supplementation treatments presented a higher protein content, possibly indicating these fish to invest more energy in their excretions. Hence, increased *Ralstonia* abundance might be related to the metabolism of nitrogenous compounds on diets with higher microalgae content.

Other *Arthrospira* supplementation studies found congruent results. The microbiota among different treatments is composed mostly of *Cetobacterium* and *Aeromonas* [39]. Interestingly, this microbial composition was present in healthy fish and fish with probiotic inclusion [39,48]. Additionally, other fish species fed with *Arthrospira* supplemented feed had a similar microbiome profile [50]. Differently, previous studies have reported a great abundance of *Escherichia*, *Propionibacterium*, *Plesiomonas* and *Deefgea* in the gut tilapia that were not seen in our results [39,42,48].

It is relevant to remark that phyla and genera microbial composition is consistent among most replicates. This provides a noticeable pattern of the microbiome of tilapia fish fry. Moreover, inclusion of *Arthrospira* species in feed is widely studied, however, its effect on gut microbiota has only few research. Hence, we expect the microbial profile presented in our study to provide helpful insight for forthcoming research.

To statistically evaluate microbial composition, alpha diversity indexes of richness (Observed and Chao1), Shannon and Simpson were calculated. The supplementation with *A. maxima* did not seem to have a significant effect in alpha diversity ($p > 0.05$). The highest diversity value across indexes was found in 15% supplementation treatment, followed by 5% (Figure 2). Contrary, diversity in 10% treatment decreased in comparison with control group, compiling the lowest values.

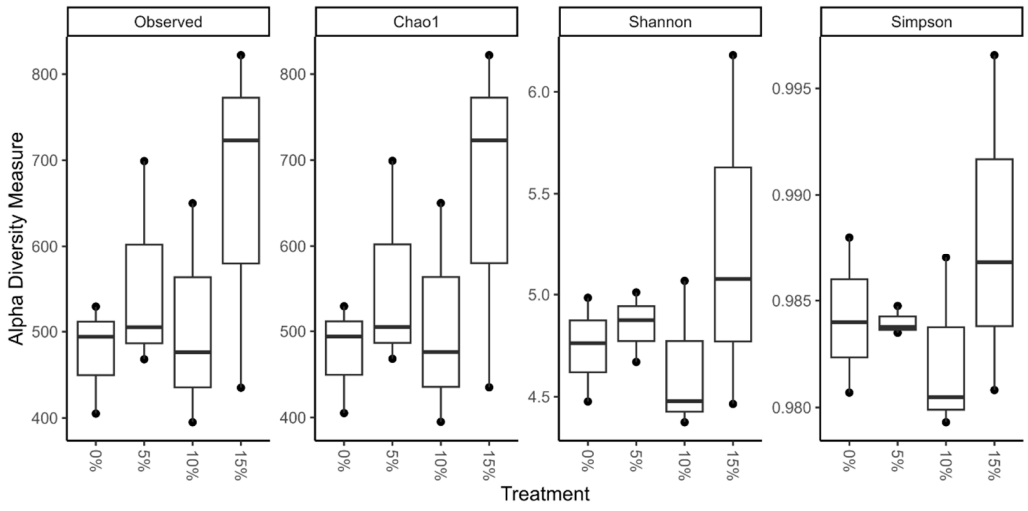

Figure 2. Alpha diversity indexes of richness (Observed, Chao1), Shannon and Simpson of the gut microbiota of tilapia fry fed with *A. maxima* supplemented feed. None of the values presented a significant difference per treatment ($p > 0.05$).

Previous studies evaluating *Arthrospira* feed supplementation had no significant difference in alpha diversity [42]. The gut microbiota of tilapia seems to be stable, as same pattern is present in studies of pre- and probiotics implementation. In this research, prebiotics had no significant effect on alpha diversity of gut microbiota whilst considerably improving

growth performance of fish [48,51–53]. Interestingly, relevant changes in alpha diversity have mostly been reported in diseased or fish exposed to pollution, where dysbiosis is reported [39,54].

Our results describe a microbiota profile similar to literature reports. This suggests that tilapia gut presents a core microbiome consistent in transcontinental fish strains [38]. The stability of this microbiota is reflected in the lack of significant differences in alpha diversity, even in treatments of supplemented feed with proven beneficial additives. Contrary, pollution and illness seemed to severely affect gut microbial composition, causing dysbiosis [55]. We considered that microalgae inclusion had no negative effect in the fish microbiota as it follows similar profiles of healthy fish. Hence, implementation of *Arthrospira maxima* avoided dysbiosis of gut microbiota. Considering the significant effect of *A. maxima* on tilapia fry proximal composition discussed in previous sections, this is a desirable outcome. Overall, both aspects favor and fundament the proposal of *A. maxima* as feed supplement for production of tilapia.

To accesses the potential metabolic function of detected taxa in gut microbiota, we predicted associated pathways using PICRUST2 [27]. The most abundant metabolic pathways were aerobic respiration I (cytochrome C) (PWY-3781), pentose phosphate (non-oxidative branch, NONOXIPENT-PWY), pyruvate fermentation to isobutanol (PWY-7111), pyruvate fermentation to acetone (PWY-6588) and acetyl-CoA fermentation to butanoate (PWY-5676) (Figure 3). The major abundance of aerobic respiration pathways in gut (an anaerobic environment), although odd, might be attributed to presence of facultative anaerobes, as described for several species of *Aeromonas* [56]. Overall, the top 50 pathways annotated were related to biosynthesis of amino acids, nucleotides, lipids, and basic metabolic processes (e.g., respiration, glycolysis) (Supplementary Table S2). Among the top 50, one of the only catabolic pathways found were related to recycling of nitrogenated bases. Interestingly, this result tracks back to the presence of *Ralstonia,* associated to this function in the GI, and a potential excess of protein in 10% and 15% inclusion trials. Remarkably, Calvin-Benson-Bassham Cycle, a metabolic route related to carbon fixation in photosynthesis, was among the most abundant. This can be associated with the presence of digested microalgae in the microbiota.

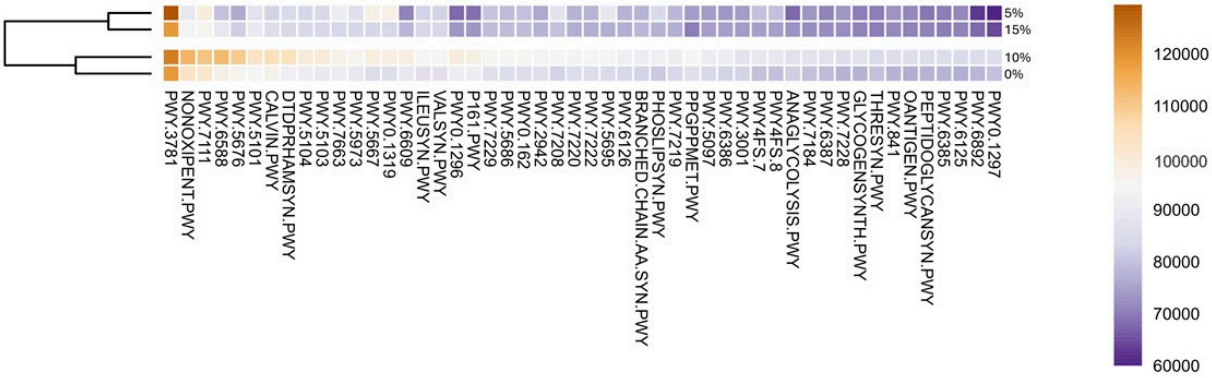

Figure 3. Top 50 most abundant predicted metabolic pathways annotated by PICRUST2. Values represent the averaged abundance of the samples triplicate. Pathway abbreviation can be found in the Supplementary Material.

The dominance of pathways related to metabolism remains unsurprising as it has been previously described in other fish species [57–59]. The supplementation of *A. maxima* had no significant effect on pathways abundances ($p > 0.05$). Although a clustering of 5% and 15% inclusion, and 10% and control group is clearly visible in the dendrogram. In this regard, 5% and 15% treatments present a lower abundance than 10% and control groups. We consider that a higher abundance of *Cetobacterium* genera in microbiota might be responsible for such results. As mentioned, *Cetobacterium* is considered part of core microbiota of freshwater fish. Previous studies have demonstrated that the genus is able

to stabilize gut microbiota, gut barrier thigh junctions and produce vitamin B12 [60]. This proposed the idea of these species as potential probiotics, with promising results enhancing health and fish growth [46,61].

Additionally, 10% and control treatments presented less variation per sample, which indicates a more stable microbiota. A study of gill microbiome demonstrated that stability of microbiota is related with less opportunistic and potential pathogenic bacteria, overall, a better health [62]. We consider that clustering and higher abundance of metabolic pathways of 10% treatment and control group might be caused by a more stable microbiome.

In summary, the 5% inclusion of *A. maxima* seems like an optimal diet for enhancing nutritional quality of tilapia fry biomass. At this percentage, the growth performance of tilapia fry is not compromised. Additionally, the overall inclusion of the microalgae favors the microbiota stability and predominance of metabolic pathways of beneficial gut bacteria. However, it is worth remembering that PICRUST2 provides bioinformatic prediction based merely on 16S rRNA genes taxonomy. Results should be interpreted with caution as it will be necessary to have a more in-depth analysis based on wet-lab experiments, metabolomics, metatranscriptomics, among others, for further validation.

3.3. Arthrospira Maxima Supplementation Treatment Effect on Gut Microbiota

We evaluated different microbiota composition per treatment using a Principal Coordinate Analysis (PCoA) (Supplementary Figure S3) and a non-metric multidimensional scaling analysis (NMDS) (Figure 4), based on Bray–Curtis distances. Our results showed a differential clustering of those samples in the control group in comparison with treatments. The gut microbiota of tilapia fry supplemented with *A. maxima* seems to be similar among treatments, as they intertwined in the clustering (Figure 4). However, no significant differences were present in Bray–Curtis distances ($p > 0.05$).

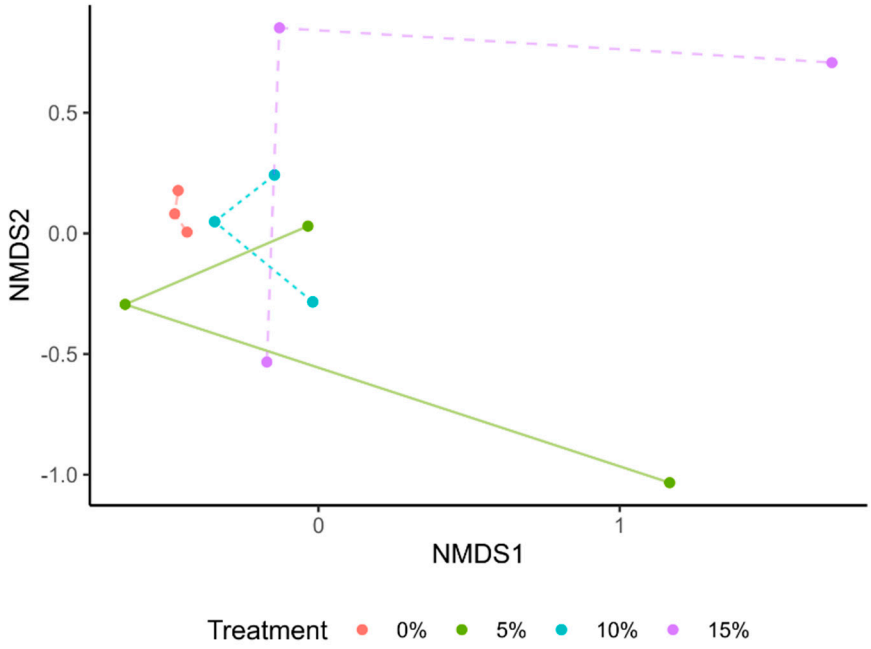

Figure 4. Non-metric multidimensional scaling (NMDS) analysis of gut microbiota of tilapia fry. None of the values presented a significant difference per treatment ($p > 0.05$).

Similarly to alpha diversity, the clustering of gut microbiota seems to lack significant differences among treatments. A systematic review recovered several tilapia gut metaamplicon taxonomies from pre- and probiotics supplementation trials [43]. The PCoA based on these sequences showed no clear grouping, as in our results [43]. However, NMDS analysis of healthy and diseased fish clustered samples differently according to health

status [39]. These outcomes support the hypothesis of our fish not being negatively affected by *A. maxima* supplementation and the prevalence of a healthy microbiota.

To have a deeper understanding of how *A. maxima* supplementation affects the microbial composition of tilapia fry's gut, we analyzed shared genera per treatment (Figure 5). A total of 63 genera were present in all samples, which might represent a core microbiome of fish gut. Interestingly, treatment-treatment samples overlap seemly present more shared taxa than treatment-control overlaps. This correlates with previous results, where treatments samples intertwined in NMDS analysis (Figure 4). Moreover, unique taxa per treatment were higher than those genera found only in the control group (Figure 5). Remarkably, the 15% supplementation treatment had 177 unique taxa. Overall, these results indicate a great variability in microbiota per treatment, with more unique genera in supplemented groups. Supplementary Table S3 encloses exclusive genera for each of the treatments.

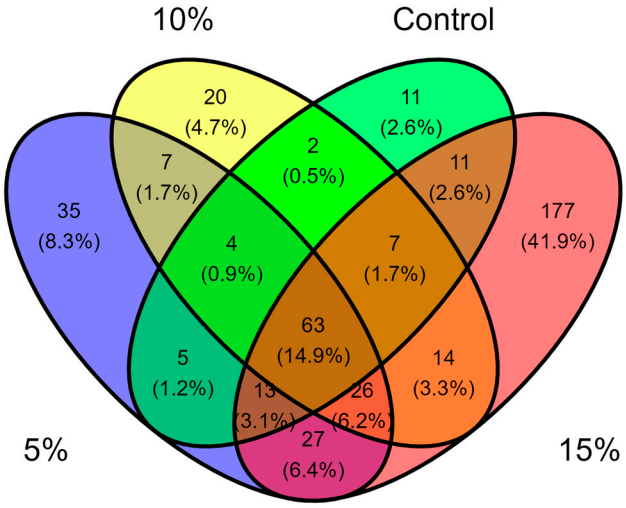

Figure 5. Genera shared among the gut of tilapia fry fed with *A. maxima* supplemented feed.

While reviewing literature, reports of functionality of some of the unique genera were found in different *A. maxima* supplementation studies. Regarding control group, *Clostridium* genus has been associated with opportunistic pathogens in microbiota [63]. In some cases, presence of *Clostridium* species is negatively correlated with production of relevant lipids, such as docosahexaenoic acid [57]. This brought the idea that fish in control group might present a higher abundance of potential pathogenic microorganisms, a hypothesis later explored.

Arthrospira maxima 5% supplementation treatment demonstrated the presence of several genera with reported positive effects in tilapia fish. *Anaeromyxobacter* is thought to be related with bioremediation of toxic compounds [64]. *Deinococcus* and *Sediminibacterium* presented a higher abundance in fish fed with probiotics [43,65]. *Oscillochloris* is a microalga used as probiotic in biofloc systems [66]. *Blastopirellula* abundance is significantly increased in fish with enhanced immune response [67]. *Kocuria* is capable of producing metabolites that inhibit tilapia pathogens [68]. Finally, *Pseudorhodoplanes* is a genus participating in the regulation of immune and metabolic tolerance under stressful conditions [69].

In 10% inclusion, the most remarkable genera with a positive impact in tilapia fish reported is *Terrimicrobium.* Previous studies have stated this bacterium to be benefited in bioflocs systems, as its relative abundance is significantly higher in fish fed with probiotics [70].

The 15% supplementation treatment although having a remarkable quantity of unique genera, only a few reported positive effects in tilapia fish. *Bifidobacterium* is commonly used as a probiotic [71]. *Cellulomonas* is thought to facilitate the digestion of cellulose by producing digestive enzymes [72]. Moreover, *Enhydrobacter* is a commensal genus

in other fish species, producing antimicrobial compounds [73]. Additionally, some of the genera exhibit a higher abundance in fish fed with probiotics (*Finegoldia, Nitrospira, Tepidimonas*) [74,75], or produce an enhanced immune response (*Rhodopirellula*) [67].

It is noticeable that supplementation treatments compose a vast variety of unique beneficial bacteria genera, whereas the control group presents only genera with unknown function and potential pathogens. As mentioned above, this brought up the hypothesis of *A. maxima* supplementation significantly decreasing relative abundance of potential pathogens. In order to evaluate this idea, we extracted the abundances of common genera with reported pathogenicity in tilapia fish. According to Haenen et al. (2023), the most relevant bacterial infection in tilapia are streptococcosis (*Streptococcus*), aeromonasis (*Aeromonas*), francisellosis (*Francisella*), columnaris (*Flavobacterium*) and vibriosis (*Vibrio*) [25].

Neither *Francisella* nor *Vibrio* genera were present in our sample. *Streptococcus* genus was only present in the 10% *A. maxima* supplementation treatment, with a relative abundance of 0.00027. Streptococcosis is mostly caused by two microorganisms: *S. iniae* and *S. agalactiae* [25], whilst other *Streptococcus* species have even been reported as common probiotics in aquaculture [76]. Hence, we consider the *Streptococcus* presence in one sample (10% inclusion, second replica) at such low abundance to represent insufficient data to form conclusions regarding fish potential health.

Aeromonas and *Flavobacterium* genera presented a very significant difference in control group compared to supplementation treatments ($p < 0.001$) (Figure 6). Previous studies have demonstrated that *Arthrospira* supplementation can enhance the immune systems of tilapia [77–79]. The microalga significantly increases total erythrocyte and leukocyte count, hemoglobin percentage, packed cell volume, immunoglobin M, lysozyme, phagocytic activity, lymphocytes, and eosinophils [77,80]. These parameters are responsible for improving immune response. Specifically, some studies have reported an increase in disease resistance against pathogens such as *Aeromonas hydrophila* and *A. veronii* in *Arthrospira* supplemented diets [81,82]. Although our results cannot ensure a decreased abundance of pathogenic *Aeromonas* and *Flavobacterium* species, we report a trend consistent with the literature.

The enhanced immunological response of tilapia with *Arthrospira*-supplemented diets may, in part, be attributed to secondary and valuable metabolites of microalgae. The presence of (C-)phycocyanin is known to stimulate erythropoietin hormone production, enhancing hematopoiesis, and building immune capacity [77,79]. Moreover, studies have hypothesized that (C-)phycocyanin and polysaccharides play a role in growing leukocytes [83]. Other pigments from *Arthrospira* (e.g., carotenoids, chlorophyll) are known to possess high antioxidant capacity, reinforcing the oxidative stress response in tilapia [84–86]. Additionally, fatty acids from *Arthrospira,* specifically linoleic and gamma-linoleic, have been positively associated with immune cell activation [87]. *Arthrospira* also contains volatile hydrocarbons (e.g., heptadecane), which have demonstrated anti-inflammatory properties [88,89].

Antimicrobials compounds are common in *Arthrospira* species, supporting its biotechnological potential. Bioassays showed high antibacterial capacity, indicating that these microalgae could be promising food additives (as tested in this study) for enhanced disease resistance [90,91]. Interestingly, *A. maxima* produces a pectin which enhances resistance against pathogenic *Aeromonas hydrophila* and *Edwardsiella piscicida* [92].

Aquaculture technique has contributed to the rise in tilapia production to fulfill the global demand. However, intensive tilapia aquaculture comes with increases stress of cultured fish leading to a higher susceptibility to infectious diseases [93]. It has been estimated that the fish farming sector loses more than USD 6 billion annually [94,95]. Tilapia are considered relatively disease-resistant, although they remain highly susceptible to the pathogenic microorganisms described above. In order to deal with this challenge, the use of microalgae as immunostimulants has been proposed [96]. This measure favors the stability of a healthy microbiota in the intestinal tract, as here depicted by the inclusion of *A. maxima* in tilapia fish diet. The presence of these commensal microbes acts as an

immune defense response by antagonizing the pathogens colonization, avoiding dysbiosis and further infections [93].

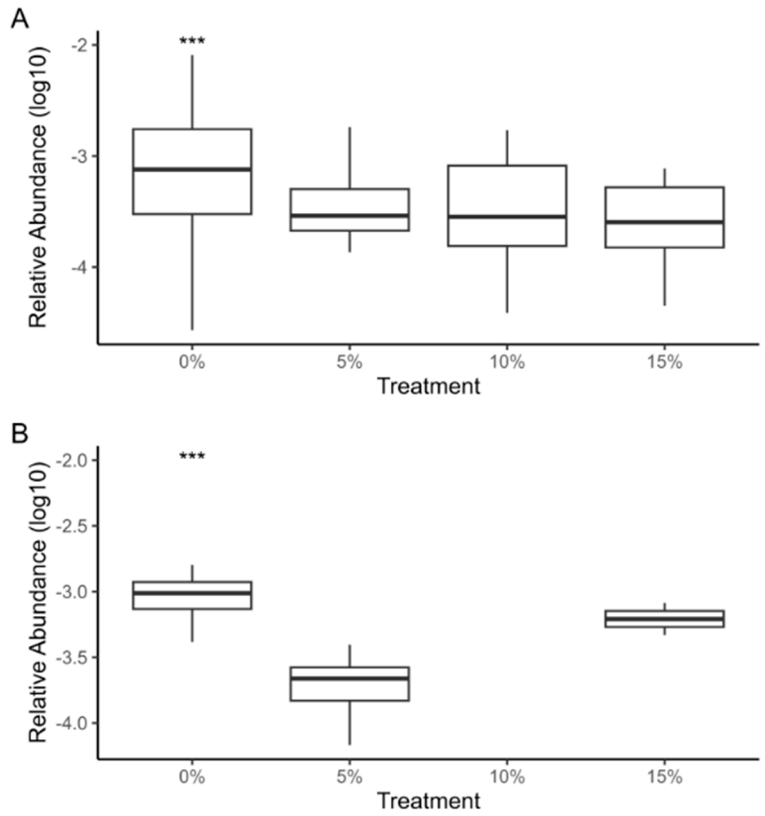

Figure 6. The logarithmic relative abundance of *Aeromonas* (**A**) and *Flavobacterium* (**B**) genera in the gut microbiota of tilapia fry fed with *A. maxima* supplemented feed. The control group presented a very significant difference ($p < 0.001$) compared to other treatments, this is represented with "***".

Previous studies evidenced that microalgae supplementation is able to significantly increase the white blood cell and platelet count, and percentage of lymphocytes in tilapia, key hematobiochemical parameters related to immune response [10]. More specifically, *A. platensis* nanoparticles inclusion significantly decreased the cumulative mortality of tilapia fish in bioassays with *Aeromonas hydrophila* infection [82]. Overall, the use of microalgae in aquaculture shows relevant results maintaining the stability of the gut microbiota as well as decreasing the severity of infectious diseases in tilapia. Hence, we consider it relevant to progressively study these beneficial microbes to develop modern solutions against the emerging challenges in aquaculture systems.

As presented, *Arthrospira maxima* supplementation improved the number of unique genera per treatment, with the highest number observed in the 15% inclusion group. Remarkably, all microbiomes with *A. maxima* supplementation exhibited unique genera with reported benefits for tilapia, whilst the control group genera had undefined functions and potential pathogens. This guides us to analyze how *A. maxima* affects the abundance of genera with potential pathogenic species. Our results showed a significant decrease in the relative abundance across all supplementation treatments. Although the presence and abundance of specific pathogenic species cannot be ensured, our results align with existing literature supporting our hypothesis. We considered, in agreement with other authors, that valuable compounds and secondary metabolites in *A. maxima* are partially responsible for decreased abundance. Additionally, the presence of beneficial genera in the microbiota composition might play a role in this dynamic.

4. Conclusions

Supplementation with *Arthrospira maxima* at 5% maintained the nutritional value of tilapia fry biomass and growth parameters comparable to the control group. Microalgae inclusion presented similar gut microbial composition to those reported for healthy tilapia and tilapia feed with pre- and probiotics. In this sense, Fusobacteria, Proteobacteria, Actinobacteria and Planctomycetota were the most abundant phyla, and *Cetobacterium*, *Pseudomonas* and *Aeromonas* the most representative genera. Functional predictions revealed a predominance of beneficial metabolic pathways with *A. maxima* supplementation. This might be associated with a more stable microbial composition in respective samples.

Microbiota composition was similar among inclusion treatments. Microalgae supplementation seems to favor the presence of beneficial species in the gastrointestinal tract. This was reflected in the description of exclusive genera per treatment, where the control group had fewer unique genera compared to *A. maxima* treatments. In this sense, the genera found in the control group had unknown functions in tilapia and included potential pathogens, while *A. maxima*-supplemented groups exhibited beneficial bacteria known to support the health of tilapia and other fish species. Further analysis showed that *A. maxima* inclusion significantly decreased the abundance of genera related to potential pathogens.

Given the emerging infectious diseases causing substantial losses to the global aquaculture sector, the continuous search for solutions remains highly relevant. The biotechnological application of microalgae and their natural products for enhancing the immune response, stabilizing the microbiota, and decreasing the severity of disease should be an area of great research interest. We propose that *A. maxima* supplementation might serve as a potential preventive measure against infectious diseases in tilapia. In this regard, a 5% *A. maxima* inclusion appears to be the optimal diet for achieving promising results.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fishes9100374/s1, Supplementary Table S1: Summary of the number of sequences during each step of the preprocessing pipeline, Supplementary Figure S1: Rarefaction curves of observed features (amplicon sequence variants, ASVs) per sample. All the analyzed samples converged before reaching the 25,000 reads, indicating that ASV richness was obtained. The 5% supplementation is represented with letter "A", 10% with "B", 15% with "D" and the control group with "C". Associated number to the sample refers to each of the triplicates, Supplementary Figure S2: Relative abundance of top 10 most abundant phyla in the gut microbiota of tilapia fry fed with *A. maxima* supplemented feed. Associated number refers to the replica, Supplementary Figure S3: Principal Coordinate Analysis of gut microbiota of tilapia fry. None of the values presented a significant difference per treatment ($p > 0.05$), Supplementary Table S2: Top 50 predicted metabolic pathways annotated with PICRUST, Supplementary Table S3: Unique genera in the gut microbiota of tilapia fry fed with *A. maxima* supplemented feed.

Author Contributions: Conceptualization, D.R.-V., O.G.-E., R.G.-W., F.M.-V., F.V.-R., F.V.-P., M.C., M.G. and K.N.-M.; methodology, D.R.-V., O.G.-E., R.G.-W., F.M.-V., F.V.-R., F.V.-P., M.C., M.G. and K.N.-M.; software, D.R.-V.; validation, D.R.-V., O.G.-E., R.G.-W., F.M.-V., F.V.-R., F.V.-P., M.C., M.G. and K.N.-M.; formal analysis, D.R.-V., O.G.-E., R.G.-W. and K.N.-M.; investigation, D.R.-V., O.G.-E., R.G.-W., F.M.-V., F.V.-R., F.V.-P., M.C., M.G. and K.N.-M.; resources, D.R.-V., O.G.-E., R.G.-W., F.M.-V., F.V.-R., F.V.-P., M.C., M.G. and K.N.-M.; data curation, D.R.-V., O.G.-E., R.G.-W., F.M.-V., F.V.-R., F.V.-P., M.C., M.G. and K.N.-M.; writing—original draft preparation, D.R.-V.; writing—review and editing, D.R.-V., O.G.-E., R.G.-W., F.M.-V., F.V.-R., F.V.-P., M.C., M.G. and K.N.-M.; visualization, D.R.-V., O.G.-E. and K.N.-M.; supervision, D.R.-V., O.G.-E., R.G.-W., F.M.-V., F.V.-R., F.V.-P., M.C., M.G. and K.N.-M.; project administration, O.G.-E., R.G.-W., F.M.-V., F.V.-R., F.V.-P., M.C., M.G. and K.N.-M.; funding acquisition, O.G.-E., R.G.-W., F.M.-V., F.V.-R., F.V.-P., M.C., M.G. and K.N.-M. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The original data presented in the study are openly available in the National Center for Biotechnology Information at PRJNA1105131 BioProject accession.

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Conflicts of Interest: The authors declare no conflicts of interest.

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