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Seasonal and spatial dynamics of the intestinal microbiome in tropical freshwater fish: insights from *Astyanax aeneus* and *Brycon costaricensis* in the Peñas Blancas river basin, Costa Rica

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Abstract

Background The intestinal microbiome plays a crucial role in fish development and health, facilitating essential functions such as nutrient uptake, immune system response, and disease resistance. However, the microbial communities of Neotropical freshwater fish, such as *Astyanax aeneus* and *Brycon costaricensis*, remain largely unexplored. Understanding how microbiomes vary in relation to environmental gradients is key to identifying potential sentinel species for ecosystem monitoring. To understand the dynamics of bacterial diversity and community structure, we collected intestinal content samples from 165 individuals of both species from six points along the Peñas Blancas river basin, Costa Rica, during the dry and rainy seasons and during an intermediate period.

Results Metabarcoding analysis of the 16 S rRNA gene revealed that the intestinal microbial communities of both species were dominated primarily by the genera *Cetobacterium*, *Clostridium*, *Romboutsia* and *Plesiomonas*. No significant differences were detected in the relative abundance of taxa, metabolic pathways or community structure between the two species and only at the Dam site, a significant increase in the Shannon index was detected in *B. costaricensis*. Conversely, distinct differences in microbial network properties were found, with *A. aeneus* showing a lower clustering coefficient and modularity, a shorter average path length and a greater number of hubs. Site and season influenced the microbial community structure of *A. aeneus* but not the relative abundance of taxa. Similarly, differentially abundant metabolic pathways, including xenobiotic degradation, were enriched in *A. aeneus*.

Conclusions The similarities in microbiome diversity and structure in both species could arise from parallels in taxonomy, habitat and diet. However, temporal and spatial shifts in the *A. aeneus* microbial community structure may be associated with sensitivity to changes in environmental stressors such as precipitation, temperature, and runoff.

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Microbial network analysis revealed that taxa in *A. aeneus* are more tightly interconnected and form fewer distinct clusters, making it a promising bioindicator for monitoring water quality and anthropogenic impacts.

Keywords *Characidae*, Tropical river, Gut, Fish, Seasons, Watershed, Metabarcoding

Background

The microbiome refers to the microbial community and its collective genomes that occupy a well-defined habitat, forming a dynamic and interactive microecosystem that is prone to change over temporal and spatial scales. This community is integrated into macroecosystems, including eukaryotic hosts, which are critical for its functioning and overall health [1]. The intestinal microbiota, which is composed of highly specialized microbial communities with a complex composition, is affected by complex interactions among microorganisms, hosts, diets, and environmental factors [2–5]. Indeed, phylogeny and the host diet are two main factors that shape the intestinal microbiome [3, 6–8]. The gut microbiome plays a critical role in host development, physiology, and health. In recent years, numerous studies have explored the gut microbiota of fish, particularly in aquaculture settings or marine species [9, 10], where microbial communities are examined in relation to diet, disease resistance, and growth performance. However, research remains limited for wild freshwater species, especially in tropical regions [11–13]. This leaves significant gaps in understanding the microbiomes of Neotropical freshwater species, which also play vital roles in ecosystem functioning [14, 15]. Microbiomes are ubiquitous, and both environmental and host-associated microbial communities respond rapidly to anthropogenic disturbances [16]. Given the sensitivity of host-associated microbiomes to environmental change, establishing baseline microbial profiles in sentinel fish species enables early detection of ecological disruption and supports biomonitoring efforts in threatened freshwater ecosystems.

Fish engage in unique interactions with their surrounding environment and the microorganisms within it, which affect their overall health and physiology [8]. They harbor large and diverse bacterial populations [17, 18], representing approximately 21 phyla [19]. The gastrointestinal microbiota of fish consists mainly of aerobic, facultative, and obligate anaerobic microorganisms [20–22], characterized by the predominance of proteobacteria such as *Aeromonas* sp., *E. coli*, *Photobacterium* sp., *Pseudomonas* sp. and *Vibrio* sp [8, 17].

The fish gut microbiome plays a critical role in host fitness, immune function, and behavior [23–25]. Host-driven mechanisms that shape microbiome composition may confer selective advantages, with microbiome variation typically being lower within species than between them. As host species diverge, their associated microbial communities also differ, mirroring host phylogeny [26, 27]. This pattern, known as phylosymbiosis, highlights the evolutionary relationship between host species and their microbiomes, providing evidence that microbial community composition is intricately linked to host evolution [27].

Although microbiome studies have been conducted in freshwater ecosystems, they are far fewer than those focusing on marine or terrestrial species are, leaving a significant knowledge gap. The intestinal microbiomes of *Astyanax aeneus* and *Brycon costaricensis*, two common species in Mesoamerican rivers, remain unexplored, as does their potential as sentinels of ecological changes in river ecosystems. Both are abundant in the Costa Rican Peñas Blancas river [28] and other neotropical streams. The first one is distributed from Mexico to Panama and is one of the most broadly distributed species in Costa Rica, ranging from coastal streams, lakes, lagoons, brackish waters, Mangrove swamps and creeks, up to elevations of 1000 m above sea level. This species can tolerate temperatures ranging from 20 to 37 °C [29]. In contrast, the second one is restricted to Nicaragua and Costa Rica and can be found in lakes, rivers and Small creeks at elevations of 0–600 m in the Atlantic and northern regions [30].

Both species are omnivorous and feed on benthic microalgae, fish, insects, leaves, and seeds [30–33]. Interestingly, the diet of *B. costaricensis* has a crucial impact on its abundance in small creeks; it is usually absent from deforested areas without fruit-bearing trees along stream banks, suggesting its potential as an indicator of stream conditions associated with deforestation [30]. Furthermore, *B. costaricensis* has undergone an ontogenetic change in the main gut enzymes associated with dietary shifts from carnivorous to mainly herbivorous [34].

On the other hand, *A. aeneus* has been used for environmental studies as a possible indicator of the presence of xenobiotics in rivers, as it can accumulate and biomagnify large quantities of metals in its tissues due to its trophic position and feeding habits [35]. It is also considered a very important species due to its high phosphorus recycling ability, especially in low-phosphorus streams [31], and its biomarkers have been used in ecotoxicological studies as indicators of water quality [36, 37]. Despite their ecological relevance, the composition and dynamics of the gut microbiome in these species remain largely unexplored.

This study aimed to investigate the variation in the gut microbiome of riverine populations of *(A) aeneus* and *(B) costaricensis* across different seasons and sampling sites in the Peñas Blancas river basin. This research provides a baseline for future research and insights into the impacts of varying degrees of human influence, such as dams, agriculture, livestock production, ecotourism, and human population density, on microbial community dynamics within this ecosystem.

Methods

Sample collection

Astyanax aeneus and *Brycon costaricensis* were collected from six sites across the Peñas Blancas river basin in San Ramón, Alajuela, Costa Rica. These sites were located in the upper (Children's Eternal Rainforest), middle (Dam and La Lucha), and lower (Chachagua, Chachagüita, and Burro Brujo) regions of the river. The sampling was conducted during three different seasons (rainy, transition (rainy-dry) and dry seasons) from 2022 to 2023 (Fig. 1). A detailed description of each area is provided in the supplementary material (Table S1, Additional file 1).

Fish collection was conducted under permits R-CM-UNA-002-2002-OT-CONAGEBIO, R-CM-UNA-008-2022-OT-CONAGEBIO, and R-CM-UNA-002-2023-OT-CONAGEBIO. Animals were captured via three- and five-meter fishing lines. At each sampling point and during each season, five individuals of each fish species were collected. The captured fish were kept alive for less than 1 h in aerated jars labeled with the species and the collection site. On the day of capture, the fish were anesthetized via a clove oil-based anesthetic (eugenol) and euthanized via cervical transection. A section of the intestine, specifically the midgut and hindgut, was then excised, weighed (average weight: 0.095 g) and stored in 2 mL conical microtubes at −40 °C for 24–36 h, depending on the capture day, in preparation for microbiome analysis. At the time of dissection, all fish presented visible intestinal contents. The samples were subsequently transported in a cooler at 4 °C for 3 h to the Regional Institute for Studies in Toxic Substances

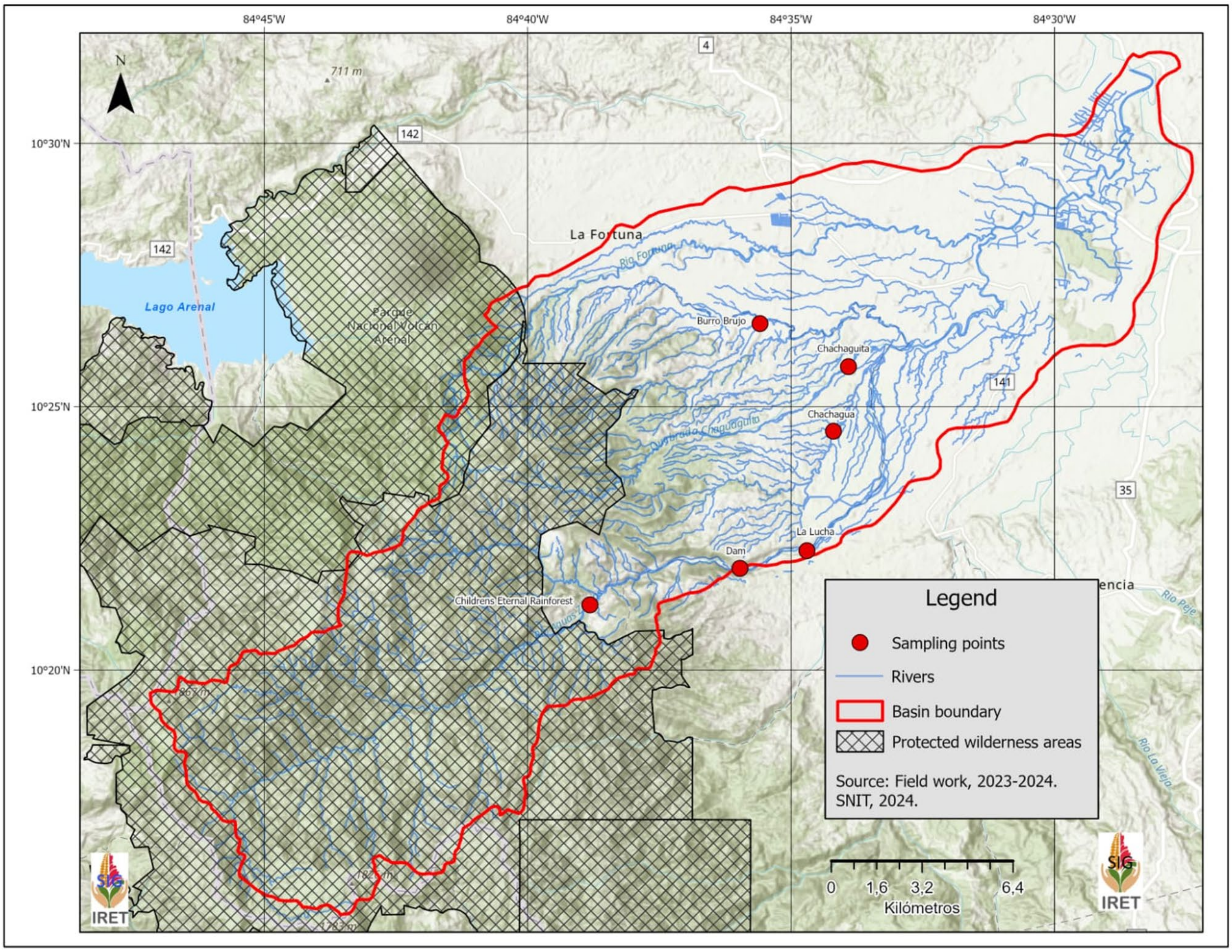


Fig. 1 Sampling points in the Peñas Blancas river basin, San Ramón, Alajuela, Costa Rica

(IRET) at Universidad Nacional, where they were stored at −80 °C until processing.

Metabarcoding

Genomic DNA was extracted via the DNeasy PowerLyzer PowerSoil Kit (Qiagen) in conjunction with the MM400 mechanical disruptor (Retsch GmbH) following the manufacturer's protocol. The concentration of the extracted DNA was quantified via spectrophotometry via a Nanodrop (Thermo Scientific). Samples with concentrations less than 1.0 ng/μL were further analyzed with a Qubit 4 fluorimeter (Thermo Scientific) and the Qubit 1X dsDNA HS assay reagent, which accurately measures DNA concentrations down to 10 pg/μL.

To ensure successful amplification, a PCR quality control test was conducted in which the V4-V5 regions of the 16 S rRNA gene were targeted with the primers Bakt-341-F (CCTACGGGNGGCWGCAG) and Bakt-805R (GACTACHVGGGTATCTAATCC) (Herlemann et al., 2011). DreamTaq PCR Master Mix 2X (Thermo Scientific) was utilized according to the manufacturer's guidelines, with 0.5 μM of each primer. The thermocycling conditions included initial denaturation at 95 °C for 4 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 48 °C for 30 s, and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min. The amplification results were evaluated via agarose gel electrophoresis.

The genomic DNA samples were stored at −20 °C and subsequently shipped to Novogene Corporation, Inc., for high-throughput sequencing of the 16 S rRNA gene V4 region on the Illumina NovaSeq 6000 platform, which produced paired-end reads of 250 bases each, with a minimum of 30,000 reads per sample. The primer and adapter sequences were trimmed via Cutadapt [38]. Amplified sequence variants (ASVs) were inferred via the DADA2 v1.32 package [39], and taxonomic assignment was performed via a Bayesian classifier [40] and the SILVA SSU r138.1 database [41], all within the R environment version 4.4.2 [42] and the RStudio integrated development environment 2024.12.0 Build 467 [43].

Inferred ASVs were processed by (a) removing ASVs classified as chloroplast, mitochondria, or eukaryote, and (b) filtering out ASVs not assigned to Bacteria or Archaea. A phylogenetic tree was constructed by placing the filtered ASVs in a reference alignment of 20,000 full 16 S rRNA genes from bacterial and archaeal genomes in the Integrated Microbial Genomes database [44], utilizing HMMER [45], EPA-ng [46] and GAPPA [47], integrated into PICRUSt2 [48]. ASVs with poor alignment to the reference were excluded from further analysis. Microbial interaction networks for each species were inferred with the Network Construction and comparison for Microbiome data (NetCoMi) package [49] and the Sparse InversE Covariance estimation for Ecological Association and Statistical Inference (SpiecEasi) package [50], using the MB method [51], with lambda.min.ratio = 1e-3, nlambda = 100, and 50 stars repetitions, to shed light on the complex interplay of the microbial communities of *(A) aeneus* and *(B) costaricensis.* Microbial interaction networks were inferred at the ASV and genus levels.

Statistical analysis

First, heatmaps were created to visualize the relative abundance at the phylum, class and genus levels via the ampvis2 package [52]. The filtered raw counts were normalized for alpha diversity analysis via ranked subsampling (SRS) [53]. Subsequently, alpha diversity index values such as Shannon [54], Chao1 [55], and Simpson [56] indices were obtained through the phyloseq v1.42.0 package [57], whereas Faith's phylogenetic diversity [58] index was calculated via the picante v1.8 package [59]. Comparisons of these indices among samples from different fish species, sites, and seasonal conditions were performed via nonparametric tests, specifically Wilcoxon and Kruskal-Wallis tests, as the data did not show normality. For bacterial taxa (genera, classes, and phyla) that presented significant differences in relative abundance across sampling sites and seasons, we employed ANOVA-like differential expression analysis (ALDEx2) designed for compositional data [60]. Benjamini−Hochberg (BH) correction was applied to control for false discovery rates, with significant differences defined by a corrected p value < 0.05 from the Wilcoxon test. For beta diversity analysis, low-abundance or nonprevalent ASVs were filtered, and only those with at least 4 reads in 5% of the samples were retained [61]. A principal coordinate analysis (PCoA) was conducted via the weighted UniFrac distance [62]. Additionally, principal component analysis (PCA) was performed by transforming the raw counts through centered log ratio (CLR) transformation and calculating Euclidean distances (Aitchison distances) [63]. To investigate differences in microbiome composition between various sampling sites and seasonal conditions, we performed permutational multivariate analysis of variance (PERMANOVA) via vegan v2.5-7 [64]. Pairwise PERMANOVA tests [65] were conducted on the full dataset (including both species) to evaluate the effects of species, season, and site. Separate analyses were also performed for each species independently to assess seasonal and spatial differences. To identify ASVs associated with each fish species, site or season, we conducted an indicator species analysis using indicspecies v1.8.0. Analyses were performed using presence/absence data and the IndVal index (func = "IndVal.g") as test statistic with 999 random permutations. To account for multiple testing, p-values were adjusted using the BH method, and ASVs with adjusted p-values < 0.05 were considered significant indicators. All the statistical analyses were conducted in the R environment and RStudio.

Functional abundance prediction

Microbiome functions were predicted via PICRUSt2 v2.5.2 [48], which estimates the functional potential of microbial communities on the based on the phylogenetic placement of ASVs onto a reference tree of sequenced genomes. Because this approach uses ~ 250 bp V4 16 S rRNA gene fragments, its phylogenetic resolution is limited, and many gut-associated microbial taxa, particularly in fish, may lack closely related reference genomes. As such, predictions of metabolic pathways should be interpreted with caution. These inferences are not a substitute for direct metagenomic sequencing and may underrepresent the functional diversity of novel or poorly characterized microbial lineages. The relative abundance of the KEGG Orthologs (KOs) was subsequently refined via ggpicrust2 v1.7.3 [66]. Differences in KEGG pathway abundances between groups were analyzed via linear models for differential abundance (LinDA) [67]. This approach provides insights into variations across predicted functional profiles. Only prokaryotic KEGG pathways related to prokaryotic metabolism (e.g., carbohydrate metabolism), environmental information processing (e.g., membrane transport), cellular processes (e.g., the cellular community) and human diseases (e.g., antimicrobial resistance) were included in the analysis.

Language editing was conducted via Grammarly and Curie.

Results

A total of 165 samples were collected from six different sites across the upper, middle, and lower parts of the Peñas Blancas River watershed during three seasons (rainy, transitional, and dry) from 2022 to 2023. Notably, *A. aeneus* was the only species found in the Children's Eternal Rainforest (Table S2, Additional file 1).

Sequencing

Among the 165 fish intestinal samples processed, 98 successfully produced V4-V5 PCR amplicons and were used for Illumina NovaSeq 6000 paired-end sequencing of the V4 region of the 16 S rRNA gene (Table S3, Additional file 1). After removing the PCR primer sequences with Cutadapt and performing quality filtering and denoising with DADA2, the dataset for *(A) aeneus* consisted of 5,901,199 sequences, with an average of 105,378 sequences per sample (ranging from 25,820 to 193,206). For *(B) costaricensis*, the dataset included 3,059,257 sequences, averaging 71,145 sequences per sample (ranging from 27,271 to 197,029 sequences). The raw sequencing data were deposited in the NCBI database under BioProject ID PRJNA1142074.

DADA2 analysis yielded a total of 12,669 amplicon sequence variants (ASVs). After filtering out ASVs assigned to chloroplasts, mitochondria, unassigned bacteria, eukaryotes, and those poorly aligned to reference sequences, 11,690 ASVs were retained. Among these, 8,879 ASVs were detected in *(A) aeneus* samples, and 5,869 ASVs were detected in *(B) costaricensis* samples. In total, 854 bacterial genera from 106 classes across 42 prokaryotic phyla were identified in *(A) aeneus*, whereas 709 bacterial genera from 80 classes across 34 prokaryotic phyla were identified in *(B) costaricensis* samples.

Bacterial community profiling and predicted functional content in *A. aeneus and B. costaricensis*

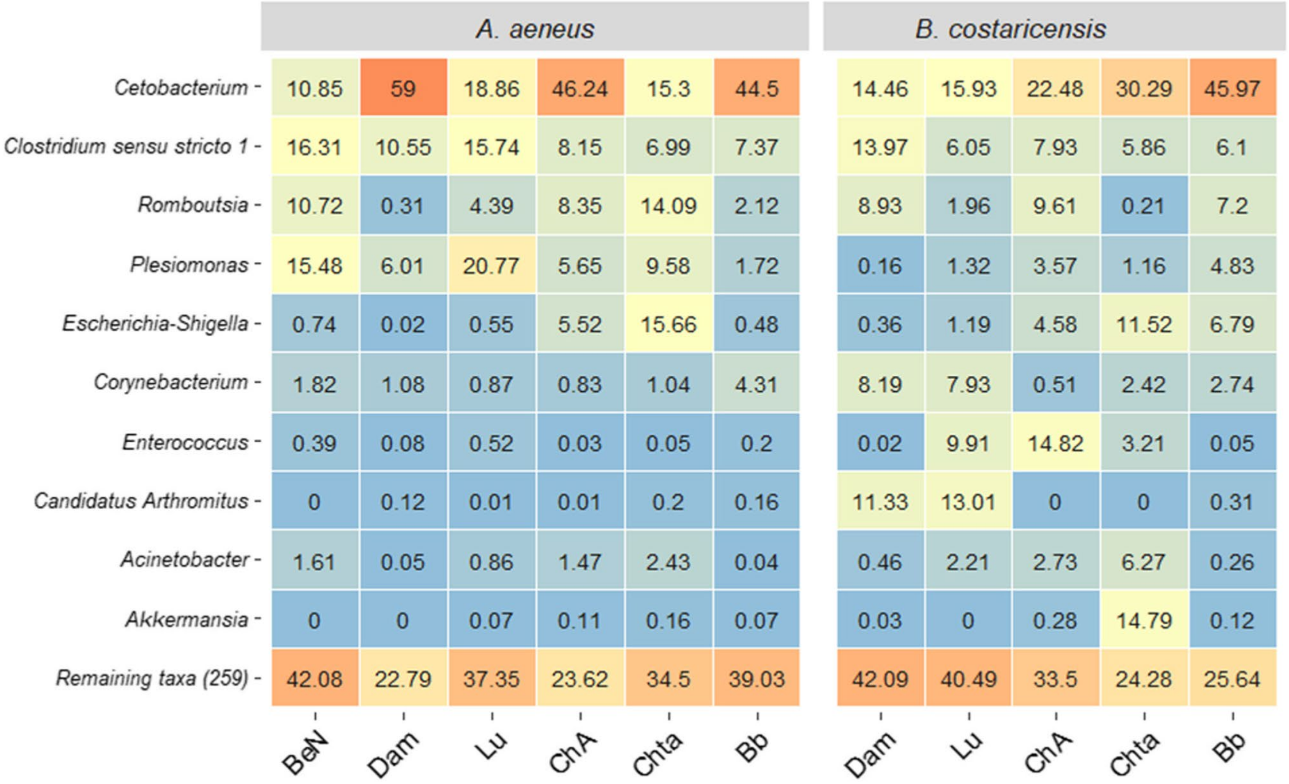
After applying abundance and prevalence filters, the intestinal microbiome of *(A) aeneus* consisted of 17 phyla, 27 classes, and 227 genera with assigned taxonomy, whereas that of *(B) costaricensis* consisted of 10 phyla, 16 classes, and 144 genera. The intestinal microbial communities of both species were dominated by the phyla Firmicutes, Fusobacteriota, Proteobacteria and Actinobacteriota (Figure S1, Additional file 2); the classes Fusobacteriia, Clostridia, Gammaproteobacteria, Bacilli and Actinobacteria (Figure S2, Additional file 2); and the genera *Cetobacterium*, *Clostridium* sensu stricto 1, *Romboutsia* and *Pleisiomonas* (Fig. 2 and Figure S3, Additional file 2).

ALDEx2 differential abundance analysis did not reveal significant differences in the relative abundance of taxa ($p > 0.05$ at the phylum, class, or genus level) between the two fish species, even when analyzed separately by season. However, when the relative abundances of taxa at each site were compared independently, the class Clostridia and the genus *Paraclostridium* were significantly ($p < 0.05$) more abundant in the *(A) aeneus* samples than in the *(B) costaricensis* samples, but only at the Chta site (Figure S4, Additional file 2).

A PICRUSt2 analysis revealed that a total of 7,039 KOs mapped to 131 prokaryotic KEGG pathways across all the samples. However, the subsequent LinDA analysis revealed no significant differences in the abundance of these pathways between the two species.

Bacterial diversity and community structure in *(A) aeneus and (B) costaricensis*

Alpha diversity indices were obtained for each sample (Table S4, Additional file 1). Compared with *(A) aeneus*, *(B) costaricensis* presented higher Shannon index values only at the dam site ($W = 7$, $p < 0.05$) (Fig. 3). However, there were no statistically significant differences in the Shannon indices between the *(A) aeneus* and *(B) costaricensis* samples ($W = 1115$, $p > 0.05$) (Figure S5, Additional



	A. aeneus						*B. costaricensis*				
	BeN	Dam	Lu	ChA	Chta	Bb	Dam	Lu	ChA	Chta	Bb
Cetobacterium	10.85	59	18.86	46.24	15.3	44.5	14.46	15.93	22.48	30.29	45.97
Clostridium sensu stricto 1	16.31	10.55	15.74	8.15	6.99	7.37	13.97	6.05	7.93	5.86	6.1
Romboutsia	10.72	0.31	4.39	8.35	14.09	2.12	8.93	1.96	9.61	0.21	7.2
Plesiomonas	15.48	6.01	20.77	5.65	9.58	1.72	0.16	1.32	3.57	1.16	4.83
Escherichia-Shigella	0.74	0.02	0.55	5.52	15.66	0.48	0.36	1.19	4.58	11.52	6.79
Corynebacterium	1.82	1.08	0.87	0.83	1.04	4.31	8.19	7.93	0.51	2.42	2.74
Enterococcus	0.39	0.08	0.52	0.03	0.05	0.2	0.02	9.91	14.82	3.21	0.05
Candidatus Arthromitus	0	0.12	0.01	0.01	0.2	0.16	11.33	13.01	0	0	0.31
Acinetobacter	1.61	0.05	0.86	1.47	2.43	0.04	0.46	2.21	2.73	6.27	0.26
Akkermansia	0	0	0.07	0.11	0.16	0.07	0.03	0	0.28	14.79	0.12
Remaining taxa (259)	42.08	22.79	37.35	23.62	34.5	39.03	42.09	40.49	33.5	24.28	25.64

Fig. 2 Relative abundance of the top 10 genera in the intestinal samples of *Astyanax aeneus* and *Brycon costaricensis* at the 6 sampling sites located in the upper Children's Eternal Rainforest (BeN), middle, Dam and La Lucha (Dam and Lu), and lower, Chachagua, Chachagüita, and Burro Brujo (ChA, Chta, and Bb) zones of the river. Taxa were selected based on overall abundance across all samples from both species

file 2), even when the indices were analyzed independently for each season (Figure S6, Additional file 2).

A PCoA based on weighted UniFrac distances (Fig. 4) revealed that samples from both fish species formed a single, overlapping cluster along the first and second axes. This lack of distinct clustering between the microbial communities of the two species was further supported by the PERMANOVA results (pseudo-F = 1.079, $R^2 = 0.011$, $p > 0.05$), indicating that there were no significant differences in community structure. However, PERMANOVA revealed significant differences in the microbial community structure according to season (pseudo-F = 4.505, $R^2 = 0.084$, $p < 0.01$). Furthermore, a pairwise PERMANOVA revealed significant differences in the microbial community structure of samples collected during the rainy season compared with those collected during the dry (pseudo-F = 6.165, $R^2 = 0.0946$, $p < 0.01$) and transition seasons (pseudo-F = 5.098, $R^2 = 0.067$, $p < 0.01$).

The indicator species analysis revealed only two ASVs significantly associated with *(A) aeneus* (BH adjusted $p < 0.05$), including ASV_113 (stat = 0.497, $p = 0.021$) and ASV_2659 (stat = 0.464, $p = 0.032$). In contrast, four ASVs were significantly associated with *(B) costaricensis*: ASV_426 (stat = 0.675, $p = 0.006$), ASV_45 (stat = 0.497, $p = 0.032$), ASV_1078 (stat = 0.476, $p = 0.032$), and ASV_255 (stat = 0.476, $p = 0.036$).

Influence of season and site on bacterial diversity, community structure and predicted functions

A Kruskal-Wallis H test revealed no statistically significant differences in the Shannon index between *(A) aeneus* samples from different sites (H(5) = 8.40, $p > 0.05$). Nevertheless, marginally significant differences were detected between seasons (H(2) = 6.23, $p < 0.05$), although a Dunn post hoc test did not reveal significant differences between any two seasons. Similarly, no statistically significant differences in the Shannon index were detected between samples of *(B) costaricensis* from different sites (H(13) = 9.13, $p > 0.05$) or seasons (H(13) = 9.13, $p > 0.05$).

PCoA with weighted UniFrac distances (Fig. 5) and Aitchison distances (Figure S7, Additional file 2), and PERMANOVA revealed significant differences in the microbial community structure of *Astyanax aeneus* samples according to site (pseudo-F = 2.091, $R^2 = 0.148$, $p < 0.05$) and season (pseudo-F = 4.082, $R^2 = 0.115$, $p < 0.01$). In addition, pairwise PERMANOVA revealed significant differences in the microbial community structure of samples collected during the rainy season compared with those collected during the dry

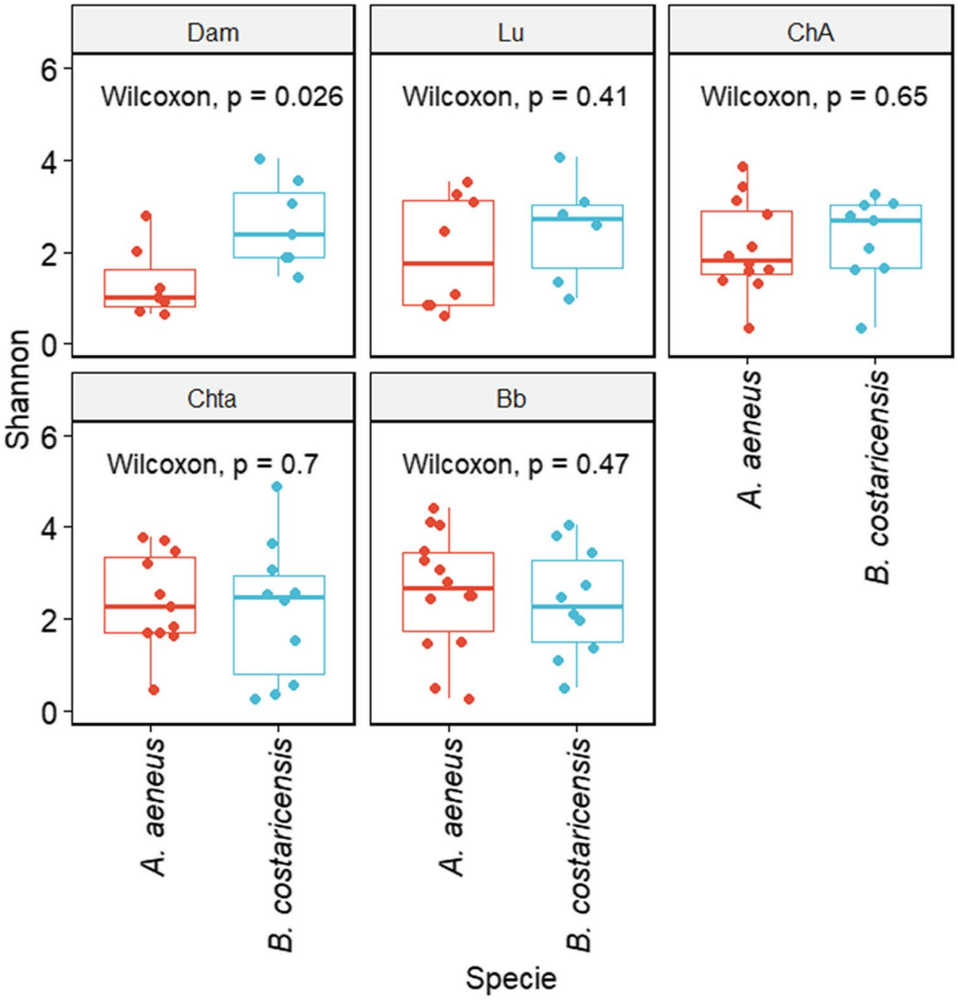


Fig. 3 Boxplot of bacterial alpha diversity, represented as the Shannon index, of the microbial communities in *(A) aeneus* and *(B) costaricensis* samples from the middle, Dam and La Lucha (Dam and Lu), and lower Chachagua, Chachagüita, and Burro Brujo (ChA, Chta, and Bb) sites of the river. A Wilcoxon test revealed significantly higher Shannon index values in samples from *B. costaricensis* than in those from *A. aeneus* at the Dam site

(pseudo-F = 5.857, R^2 = 0.151, $p < 0.01$) and transition seasons (pseudo-F = 3.893, R^2 = 0.087, $p < 0.05$).

Conversely, a PCoA based on weighted UniFrac distances (Fig. 6) did not show clear clustering of the *B. costaricensis* samples from different sites and seasons, and a similar outcome was observed when Aitchinson distances were used (Figure S8, Additional file 2). This outcome was supported by a PERMANOVA between sites (pseudo-F = 1.056, R^2 = 0.0989, $p > 0.05$) and seasons (pseudo-F = 1.245, R^2 = 0.058, $p > 0.05$).

In both the *(A) aeneus* and *(B) costaricensis* samples, ALDEx2 differential abundance analysis revealed no significant differences in the relative abundance of taxa (at the phylum, class, or genus level) between sites or across seasons.

A PICRUSt2 analysis predicted the presence of 7169 functional KOs mapped to 132 prokaryotic KEGG pathways across all *A. aeneus* samples. LinDA revealed 6 pathways that were more abundant at the BeN site than at the ChA site ($p < 0.05$) (Table S7, Additional file 1). Similarly, five of these pathways were more abundant at the Chta site than at the ChA site ($p < 0.05$). Additionally, five pathways were more abundant at the Lu site than at the ChA site ($p < 0.05$). Seasonal analysis revealed 22 pathways with significant differences in abundance between the dry and rainy seasons ($p < 0.05$) (Table S8, Additional file 1). Most of these pathways were more abundant in the dry season samples than in the rainy season samples, and only two pathways were more abundant in the rainy season samples. In contrast, only one pathway demonstrated greater abundance in the dry season than in the transition season ($p < 0.05$).

Across all *B. costaricensis* samples, PICRUSt2 predicted the presence of 6735 KOs, which were mapped to 131 prokaryotic KEGG pathways. LinDA did not identify pathways that were differentially abundant between sites. Analysis of the seasonal variations revealed that only three pathways were more abundant during the dry

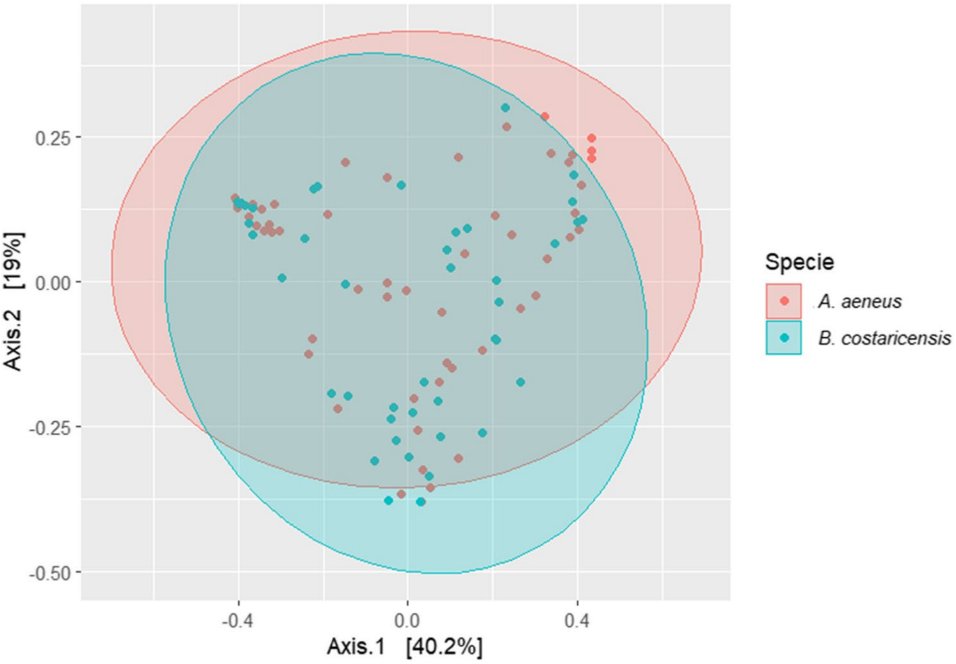


Fig. 4 Principal coordinate analysis (PCoA) based on weighted UniFrac distances from the relative abundance of the ASV in *Astyanax aeneus* and *Bycon costaricensis*. The distributions of the samples are shown in the first two axes of the PCoA, which together explain 59.2% of the variation. Points represent samples, and color indicates species. Ellipses were calculated and drawn with a 0.95 confidence level

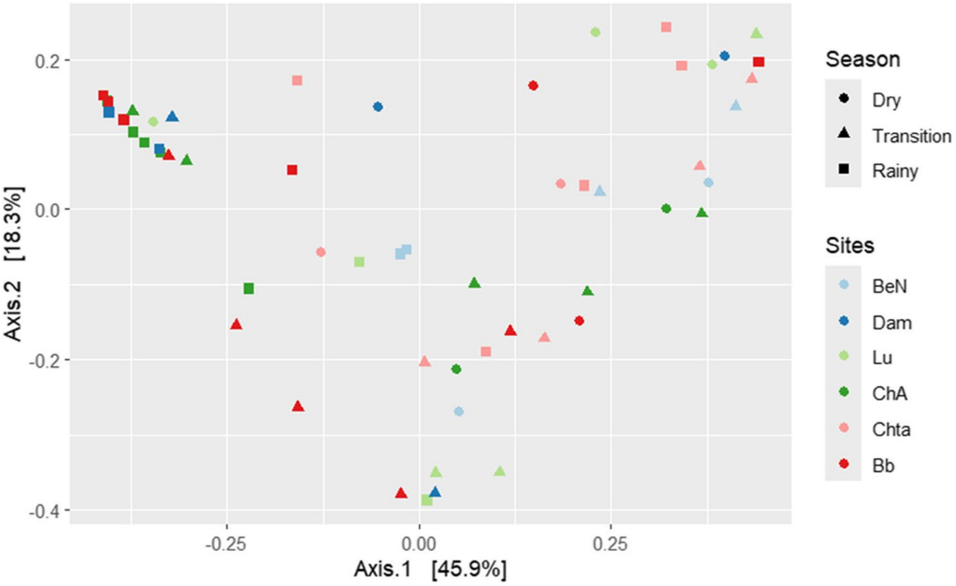


Fig. 5 Principal coordinate analysis (PCoA) based on weighted UniFrac distances from the relative abundance of ASVs in *Astyanax aeneus*. The distribution of the samples is shown on the first two axes of the PCoA, which together explain 64.2% of the variation. Colors indicate sites in the basin: upper (Children's Eternal Rainforest -BeN-), middle (Dam and La Lucha -Lu-), and lower (Chachagua -ChA-, Chachagüita-Chta- and Burro Brujo -Bb-), while the shapes indicate seasons

season than during the rainy season ($p < 0.05$) (Table S9, Additional file 1).

The indicator species analysis revealed that no ASVs were significantly associated with site in both species. However, eight ASVs significantly associated with season in *(A) aeneus* samples (BH adjusted $p < 0.05$), including six ASV associated with rainy season (ASV_99, ASV_1723, ASV_82, ASV_351, ASV_67, and ASV_250), one with dry season (ASV_301) and one with transition season (ASV_12). In contrast, no ASVs were significantly associated with season in *(B) costaricensis*.

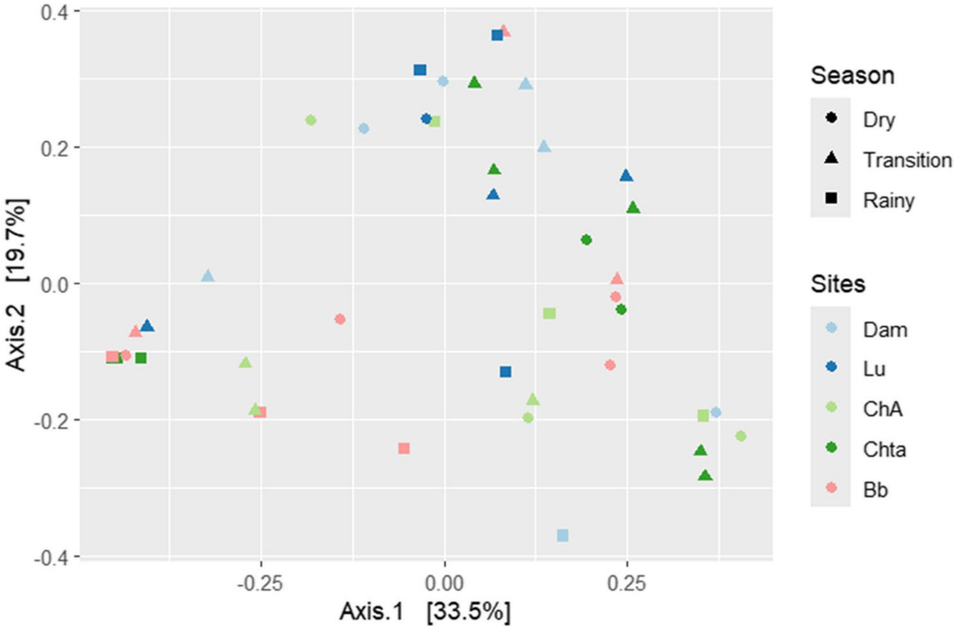


Fig. 6 Principal coordinate analysis (PCoA) based on weighted UniFrac distances from the relative abundance of the ASVs in *Brycon costaricensis*. The distribution of the samples is shown on the first two axes of the PCoA, which together explain 53.2% of the variation. Colors indicate the following sites within the basin: the middle (Dam and La Lucha -Lu-) and lower (Chachagua -ChA-, Chachagüita-Chta- and Burro Brujo -Bb-), while the shapes indicate seasons

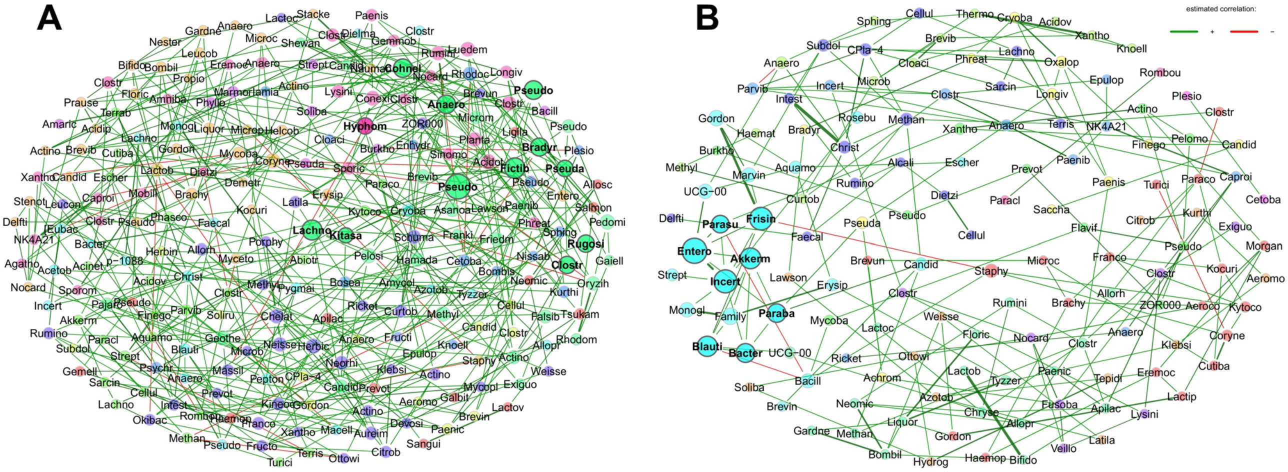


Fig. 7 Microbial interaction networks of the gut microbiomes of *Astyanax aeneus* (**A**) and *Brycon costaricensis* (**B**) show clear differences in structure and connectivity. Nodes represent bacterial genera, green lines represent positive associations between nodes, and red lines indicate negative associations. Node colors represent the detected clusters, node sizes are scaled by eigenvector centrality, and hub nodes are highlighted

Microbial interaction networks in *(A) aeneus and (B) costaricensis*

At both the genus level (Fig. 7 and Table S5, Additional file 1) and the ASV level (Table S6, Additional file 1), the microbial network properties of these species distinctly differ in structure and connectivity. At the genus level, the *(A) aeneus* network had a clustering coefficient of 0.116, which was lower than that of *(B) costaricensis* (0.179), suggesting that the microbial taxa in *A. aeneus* interact less cohesively and are more loosely structured. The modularity of the *(A) aeneus* network was 0.568, which was also lower than that of the *(B) costaricensis* network (0.727), indicating that the microbial community of *A. aeneus* was less compartmentalized into clear subcommunities. A high percentage of positive edges were found in *(A) aeneus* (95.48%) and *(B) costaricensis* (97.17%), implying that cooperative interactions dominated both networks. Both species networks had similar edge densities (0.021–0.022), indicating similar sparsity, but *(A) aeneus* presented a shorter average path length (2.662) than did *(B) costaricensis* (3.902), suggesting more direct microbial interactions in *A. aeneus*.

In terms of connectivity, while both networks had identical vertex and edge connectivity, *B. costaricensis* had marginally greater natural connectivity (0.009 vs. 0.006) than *A. aeneus*. The *(A) aeneus* network consisted of 10 clusters, with node sizes ranging from 8 to 35, whereas *(B) costaricensis* presented 11 smaller clusters, ranging from 2 to 21 nodes. Distinct hub ASVs and genera were identified in each species, with no overlap between *(A) aeneus* (12 hub genera) and *(B) costaricensis* (8 hub genera). Hubs in *(A) aeneus* included genera such as *Anaerocolumna*, *Bradyrhizobium*, and *Clostridium*. In contrast, *(B) costaricensis* hubs included *Akkermansia*, *Bacteroides*, and *Blautia*. In *(A) aeneus*, the degree of the top five hubs ranged from 13 – 10, exceeding that of *(B) costaricensis* (7 – 6), suggesting more dominant hub nodes in *(A) aeneus*. Betweenness centrality was greater in *(B) costaricensis* (0.158 to 0.108) than in *(A) aeneus* (0.065 to 0.040), indicating that the hub nodes in *(B) costaricensis* play a more critical role in connecting different parts of the network. Conversely, the closeness centrality was greater in *(A) aeneus* (0.534 to 0.505) than in *(B) costaricensis* (0.423 to 0.403), highlighting that the hub nodes in *A. aeneus* are more centrally positioned within the network.

Discussion

The intestinal microbiomes of *Astyanax aeneus* and *Brycon costaricensis* were predominantly composed of Firmicutes, Fusobacteria, Proteobacteria, and Actinobacteriota. This composition is consistent with the relative abundance of bacterial phyla typically reported in the gut microbiota of freshwater fish [19]. The most abundant genus in both fish species was *Cetobacterium*. This genus is also abundant in many freshwater fish species, such as *Oreochromis niloticus*, *Cyprinus carpio*, *Arapaima gigas*, *Lepomis macrochirus*, *Micropterus salmoides* and *Ictalurus punctatus* [68, 69]. This bacterium is a vitamin B-12 producer [68–71] that plays an essential role in animal physiology, e.g., helping in the normal function of the nervous system and the maturation of blood cells. In zebrafish under omnivorous and herbivorous diet, enriched *Cetobacterium somerae* increased acetate production and contributed to homeostasis and the improvement of carbohydrate utilization in fish [24], yet there is no evidence of carbohydrate fermentation nor data on the levels of short-chain fatty acids (SCFA) in the gut of *(A) aeneus* and *(B) costaricensis*, and to date acetate production in the hindgut appears to be negligible in freshwater fishes, as measured SCFA concentrations in freshwater fish guts are orders of magnitude lower than in specialized hindgut fermenters like kyphosid fish and most SCFA detected may come from non-fibrous substrates [72]. The high abundance of *(C) somerae* in the gastrointestinal tract of carnivorous and omnivorous species suggests a link to the trophic level [70]. In this sense, our fish species have an omnivorous diet, which can explain its high abundance.

In general, no significant differences in the relative abundance of taxa and KEGG pathways, as well as in the alpha and beta diversity metrics were detected between the two fish species, suggesting that other factors may play a more significant role in shaping their intestinal microbial communities. Only at the Dam site, the Shannon indices were greater in the *B. costaricensis* samples than in the *(A) aeneus* samples. This result may be related to the fact that *(B) costaricensis* usually feeds on fruits and insects that fall from the riverside forest, which can generate a more diverse microbiome. Like in stickleback species (Gasterosteidae), this broader niche suggests that a more diverse diet positively influences the diversity of the microbiome [73], though some of this diversity may reflect transient taxa ingested with food rather than active gut colonizers [74]. Therefore, the results obtained at this point suggest that host species and environmental factors collectively influence the composition and diversity of the fish gut microbiome [73, 75–77].

Seasonality significantly influenced beta diversity, suggesting that temporal environmental changes, such as fluctuations in temperature, rainfall, or resource availability, exert a stronger influence on gut microbial community structure than spatial location or host identity [78]. This finding aligns with those of previous studies, which highlighted that microbial community composition can be shaped by fish's taxonomic classification, trophic level, dietary preferences, and ecological niche [75–77]. The overlapping microbial community structure is in accordance with other authors who reported that host divergence had little influence on the gut microbiota of fish and that host habitat is the major determinant [19]. In this study, both species belong to the order Characiformes and develop under the same environmental conditions.

The absence of significant differences in predicted metabolic pathways between *(A) aeneus* and *(B) costaricensis* may be due to several factors. First, PICRUSt2 relies on the availability and quality of reference genomes to infer functional potential from 16 S rRNA data, and many microbial taxa present in the gut microbiota of these fish likely lack closely related representatives in genomic databases. This limits the sensitivity and accuracy of the functional predictions, particularly when comparing closely related taxa. Second, the fish used in this study were collected from different sites and during different seasons, which may have introduced environmental and temporal variability that obscures potential interspecies differences.

When analyzing samples within each species across sites and seasons, we detected some significant differences. While no significant differences in the relative abundance of taxa were detected in both species, in *A. aeneus* differences in predicted pathways were identified. At the ChA site, some metabolic pathways were less abundant compared to other sites. Alpha-linolenic acid (ALA) metabolism serves as a precursor to jasmonic acid in plants, which are consumed by some insects or prey rich in ALA [79, 80]. The variation in the abundance of this metabolic pathway between sites in *A. aeneus* could be related to the fact that the diets at BeN, Chta and Lu are possibly richer in insects or prey that consume ALA than those at the ChA site. Additionally, the biosynthesis of nonribosomal peptides of the siderophore group suggests an adaptation to an environment of low iron availability. ALA metabolism and the biosynthesis of siderophores influence the gut microbiome and its own metabolic processes, indicating that BeN, Chta and Lu could have less iron availability than ChA [81–83]. These findings highlight the interactions among dietary input, environmental conditions and microbial metabolism in shaping the gut microbiome of fish.

Cytochrome P450 (CYP) enzymes play crucial roles in the detoxification of xenobiotics in aquatic organisms, including fish, where biomarkers such as ethoxyresorufin-O-deethylase, 7-methoxyresorufin O-demethylation, and ethoxycoumarin O-deethylation are used to evaluate their activity [84, 85]. These enzymes are inducible in tissues such as the gills and intestines, where they play a role in the metabolism of xenobiotics and endogenous compounds (steroids and fatty acids) [86]. In studies involving polycyclic aromatic hydrocarbons, the first step in the biotransformation of these chemicals by certain bacteria is the generation of dihydrodiols through their oxygenase activity. In fish, CYP isoenzymes carry out a similar process. This similarity in biotransformation between bacteria and fish suggests a possible symbiotic toxicokinetic and toxicodynamic interaction between gut bacteria and their fish hosts. Some bacteria that have been identified in this process include *Aeromonas allasaccharophila*, *A. eucrenophila* and *Pseudomonas flavescens* [87]. The differential abundance of these metabolic pathways in *A. aeneus* collected at the BeN, Chta and Lu sites, compared with ChA, can be evaluated as an indicator of the presence of xenobiotics, considering that the induction of CYPs is used as a biomarker in the fish cellular system to indicate exposure to contaminants [88].

Another xenobiotic metabolic pathway, caprolactam degradation, was more abundant in samples from *A. aeneus* collected at the BeN, Lu, and Chta sites than at the ChA site. These findings suggest the potential presence of this compound, inferred from predicted metabolic pathways. While caprolactam is commonly used in nylon-6 production, and no such industry exists in the basin, caprolactam and similar compounds could originate from synthetic fibers or plastics found in wastewater, influencing bacterial communities such as *Acinetobacter*, which is known for caprolactam degradation [89, 90]. However, chemical analyses are needed to confirm the presence of such pollutants in the environment.

The inositol phosphate system is part of a coordinated metabolic response that encompasses growth, nutrient sensing and the maintenance of cellular energy stores due to its involvement in all energy processing decisions made by cells [91]. This organic phosphorus compound can be found in sediments or synthesized by some aquatic organisms, such as *Spyrodela polyrzha* [92]. In the case of *A. aeneus* samples collected at the Lu site, the increased abundance of this pathway compared with that at the ChA site may be associated with the greater availability of phosphorus from poultry production-associated wastes [93].

In *(A) aeneus* samples, 22 metabolic pathways were more abundant in the dry season than in the rainy season, whereas only one pathway was more abundant in the rainy season. Most of these pathways are involved in the metabolism of amino acids, carbohydrates, glycan biosynthesis, lipids, terpenoids, and polyketides; xenobiotic degradation; and the biosynthesis of cofactors and vitamins. For *(B) costaricensis*, three functional pathways related to the metabolism of carbohydrates, vitamins and cofactors, as well as terpenoids and polyketides, were more abundant during the dry season than during the rainy season. As both species have an omnivorous diet, soluble carbohydrate metabolism enzymes are expected to be more abundant in their digestive tract. This finding may also be associated with the phylum Firmicutes, whose members are known autochthonous gut microbiota of the freshwater herbivorous and omnivorous fish and are capable of secreting amylase and cellulase [94]. However, given the lack of specialized gut morphology and fast gut throughput times in freshwater species studied to date, the presence of amylolytic or cellulolytic bacteria does not indicate a significant role for these organisms in the digestion of cellulose and other refractory polysaccharides using fermentative pathways within the intestinal tract [72, 95]. *Cetobacterium*, a genus highly abundant in both fish species, can contribute to host nutrition via fatty acid and vitamin metabolism [96]. On the other hand, *Romboutsia* was more abundant during the dry season, indicating that the intestinal microbiome exerts some effects on host nutritional metabolism [97, 98].

The microbiome species present in the gut system have the capacity to produce different biochemical compounds, such as short-chain fatty acids, vitamins, and amino acids, while also cooperating with the host's intestinal immune system. Consequently, the fish host genome plays a crucial role in the selection of specific microbiome species, thereby shaping the overall composition of the gut microbiome [99]. However, even though functional analysis is essential for understanding the relationships between bacterial communities and host functions in fish digestion and metabolism, functional potential is predicted via databases, such as Kyoto Encyclopedia of Genes and Genomes (KEGG), which are based on reference bacterial genomes; therefore, less-studied fish gut microbes may not be well represented or lack close genomic representatives. The lack of information about these pathways can be a limitation but also an opportunity for further studies involving functional metagenomics and genome-resolved approaches, such as the reconstruction of metagenome-assembled genomes, to improve the taxonomic and functional resolution of microbial communities in non-model organisms like freshwater fish.

A. aeneus samples presented a decrease in the Shannon index during the rainy season compared to the dry and transitional seasons. One contributing factor to this decrease could be the increased rainfall during the rainy season, which leads to rivers receiving runoff from surrounding agricultural fields that contain pesticides and fertilizers, thus changing the physicochemical characteristics of the water [100]. These chemical compounds could affect the microbial diversity of *(A) aeneus*. Conversely, no significant differences in alpha diversity indices between seasons were found in the *(B) costaricensis* samples. This stable alpha diversity throughout seasons suggests that external factors do not significantly perturb the alpha diversity of the *B. costaricensis* gut microbiome [78]. These findings underscore the complex interplay between seasonality, environmental factors, and microbial diversity in shaping the gut microbiomes of both species in the Peñas Blancas river basin.

PERMANOVA results revealed significant shifts in the microbial community structure of *(A) aeneus* across sites and seasons. In contrast, no significant differences were detected in the microbial communities of *(B) costaricensis* across sampling sites or seasons. These findings suggest that the gut microbiome of *B. costaricensis* is relatively more uniform across spatial and temporal gradients compared to that of *A. aeneus*. While spatial and seasonal effects on the gut microbiome of *A. aeneus* were detected in this study, they were less pronounced than other surveys conducted in regions with more distinct seasonal variability [78, 101]. These studies highlighted how changes in precipitation, temperature, and food resources during seasons could drive shifts in the microbial community. In contrast, tropical regions typically experience less variability between dry and rainy seasons, where precipitation is a dominant seasonal factor even during supposed dry periods, in addition to the fact that all samples originated within the same river basin.

The seasonal dynamics of tropical rivers, which are influenced primarily by flow changes, affect various ecological aspects, including microbial communities [102]. The relatively stable microbial community structure observed in *B. costaricensis* may reflect consistent environmental conditions, such as stable precipitation patterns and food availability, which support a more stable microbial community structure throughout the seasons. But could also reflect the ingestion of a smaller range of allochthonous bacterial taxa associated with food.

The microbial interaction network analysis revealed differences between the networks of *(A) aeneus* and *(B) costaricensis*, likely reflecting variations in their ecological roles and gut environments. The lower clustering coefficient and modularity observed in *(A) aeneus* suggest weaker local interactions between taxa than in *(B) costaricensis*, indicating a less specialized microbial community characterized by loosely connected subgroups and less compartmentalization into clear subcommunities. In addition, the smaller average path length in *(A) aeneus* indicates a more cohesive network, which is sometimes linked to greater susceptibility to node losses [103]. All these findings point to a more stable network in *(B) costaricensis*, limiting shifts in community composition. In addition, the non-overlapping hub genera and ASVs identified in *(A) aeneus* and *(B) costaricensis* suggest contrasting microbiome organization between species. These hub taxa, which are central to microbial network structure, may drive differences in community composition and potential function, regardless of their overall abundance across space and time [104].

Owing to the interactions within the microbial communities of *(A) aeneus* and *(B) costaricensis*, along with the greater susceptibility of *A. aeneus* to environmental changes in the Peñas Blancas River across different seasons and sampling points, *A. aeneus* could serve as a potential environmental bioindicator. Environmental pollution negatively impacts microbial communities by altering species abundance and causing dysbiosis [105], as evidenced by both species showing pathogenic bacteria associated with human contamination. Additionally, inferred metabolic pathways suggested a potential capacity for xenobiotic degradation, which may reflect exposure to environmental contaminants. However, inferred pathway abundances do not confirm gene expression or activity in the gut, and may not even be present in the microbial genomes of these species, reflecting differences in related taxa rather than true functional shifts, particularly given that many gut-associated taxa in *(A) aeneus* and *(B) costaricensis* may lack close representation in current databases. Further studies should apply metagenomics or metatranscriptomics to confirm functional differences and to establish a causal relationship between pollutants in the basin and their interactions with the microbiome.

Conclusions

Astyanax aeneus and *Brycon costaricensis* exhibit similar microbial community structures and diversities, likely due to their shared habitat characteristics, comparable environments and similar diets, which shape their microbial communities in analogous ways. In *A. aeneus*, spatial and seasonal variations also influenced microbial community structure, driven by factors such as temperature, precipitation, runoff, and food availability in tropical basins. This could be due to the loosely connected taxa and less structured network, less clear subgrouping and a more compact network in *A. aeneus*, as revealed by microbial network analysis. The dominant genera in both species, *Cetobacterium*, *Clostridium*, and *Romboutsia*, are typically associated with herbivorous and omnivorous diets, underscoring the influence of diet and environmental conditions on microbial composition.

The predicted functional analysis revealed several differentially abundant metabolic pathways between the sampling sites, but only in the *A. aeneus* samples, suggesting that this species may be more responsive to factors such as xenobiotics or habitat degradation, indicating functional resilience and adaptability. This makes the *A. aeneus* microbiome a potential biomarker of xenobiotic exposure, which is why laboratory studies should be conducted to evaluate its suitability as a bioindicator of contamination in Costa Rican river basins.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-025-04279-8.

Additional file 1. Supplementary tables, Description of data: Sampling areas (Table S1), number of samples (Table S2), number of sequenced samples (Table S3), alpha diversity indices (Table S4), network properties at the genus level (Table S5), network properties at the ASV level (Table S6), differential abundance analysis of Astyanax aeneus by site (Table S7), differential abundance analysis of Astyanax aeneus by season (Table S8), and differential abundance analysis of Brycon costaricensis by season (Table S9).

Additional file 2. Supplementary figures, Description of data: Heatmap of the relative abundance of the top 10 phyla (Figure S1), Heatmap of the relative abundance of the top 10 classes (Figure S2), Heatmap of the relative abundance of the top 10 genera by season (Figure S3), Differential abundance of Clostridia (Figure S4), Boxplot of the bacterial Shannon index (Figure S5), Boxplot of the bacterial Shannon index by season (Figure S6), Principal component analysis (PCA) based on Aitchinson distances in ***Astyanax aeneus*** (Figure S7), and Principal component analysis (PCA) based on Aitchinson distances in ***Brycon costaricensis*** (Figure S8).

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Authors' contributions
M.C.M. and J.M.L. contributed to data acquisition, analysis, and interpretation, and were major contributors to manuscript writing. F.Q.A. contributed to conceptualization, study design, data acquisition, analysis, and interpretation, and was a major contributor to manuscript writing. K.B.P. made substantial contributions to funding acquisition, conceptualization, and study design, participated in data analysis and interpretation, and was a major contributor to manuscript writing. F.S.C. contributed to funding acquisition, data acquisition, analysis, and interpretation, and was a major contributor to manuscript writing. All authors read and approved the submitted version.

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Data availability
The dataset supporting the conclusions of this article is available in the NCBI database, BioProject ID PRJNA1142074, https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1142074.

Declarations

Ethics approval and consent to participate
All experimental procedures involving fish were conducted in accordance with the International Council for Laboratory Animal Science (ICLAS) Ethical Guidelines for Researchers and the Guidelines for the Euthanasia of Animals: 2020 Edition by the American Veterinary Medical Association (AVMA). Only qualified personnel with appropriate training, licensed veterinarians, were permitted to perform or participate in animal handling and experimental procedures.

Consent for publication
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Competing interests
The authors declare no competing interests.

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