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PROOF
SHORT PAPER

The Sample Method Matters When Studying Fish Intestinal Contents of Microeukaryotic Communities

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Abstract

Research on gut microbiota has primarily focused on bacteria, though eukaryotic microorganisms also play important roles in their host. In fish, the suitability of fecal sampling methods for studying these gut microorganisms remains unclear. This study aimed to evaluate two fecal sampling methods, intestinal scraping and aquarium-bottom sampling, on the diversity and composition of gut microeukaryotes in Nile tilapia (*Oreochromis niloticus*), highlighting the importance of methodological choices in microeukaryotic studies. Results showed that intestinal scraping provides a more accurate representation of the stable, resident community, avoiding potential bias and emphasizing the critical importance of methodological choices in microeukaryotic research.

Introduction

The gastrointestinal (GI) microbiome of fish is a complex ecosystem comprising bacteria, archaea, viruses, and microeukaryotes, including fungi, protists, and helminths. While most studies have emphasized the bacterial component, recent research highlights that eukaryotic microorganisms also play critical roles in digestion, nutrient transformation, immune modulation, and pathogen resistance (Bass & del Campo, 2020; Laforest-Lapointe & Arrieta, 2018; Vargas-Albores et al., 2023). In fish microbiota, the significant activity of these microeukaryotes in fish development is due to the enzyme contribution they provide for digestion (Banerjee & Ghosh, 2014), yet it remains underexplored compared to their prokaryotic counterparts.

With the advent of high-throughput DNA sequencing and targeted amplification of marker genes such as the 18S rRNA, the ability to accurately characterize microeukaryotic diversity in the fish gut has greatly improved (Elsaied et al., 2019; Watanabe et al., 2021). However, a major bottleneck persists in the comparability and accuracy of microbiota datasets: the method of sample collection. Evidence indicates that microbial profiles (including eukaryotic taxa) may vary substantially depending on whether feces are obtained directly from the gut or indirectly from the aquatic

environment (Elsaied et al., 2019; Watanabe et al., 2021). This is particularly relevant for eukaryotes, whose community structure is more sensitive to environmental exposure, host-derived factors, and sampling-induced biases.

In fish microbiome research, both invasive (e.g., scraping intestinal content) and non-invasive (e.g., collecting feces from the tank bottom) methods are employed. Invasive approaches enable targeted sampling of intestinal compartments (Ruiz, Gisbert, et al., 2024; Spilsbury et al., 2022), while non-invasive methods are more practical and ethically advantageous, minimizing stress and allowing repeated measures (Thormar et al., 2024). Yet, whether both approaches provide comparable resolutions, particularly for eukaryotic microorganisms, remains unclear. Although some bacterial studies support fecal sampling as a non-lethal proxy for gut composition A. Härer and D. J. Rennison (2023), microeukaryotic communities may respond differently to post-excretion environmental interactions or degradation.

Thus, understanding the extent to which sampling method affects the detection and interpretation of gut microeukaryotic communities is fundamental for achieving data consistency and biological relevance. Non-standardized collection strategies could lead to misinterpretation of eukaryotic profiles. This study aims to evaluate the influence of two different sampling methods on the observed diversity and composition of gut microeukaryotes in the Nile tilapia (*Oreochromis niloticus*), used as a model, and to emphasize the importance of careful methodological choices when characterizing the fish gut eukaryome.

Methods

Experimental Design

A bioassay was carried out in the aquaculture laboratory at the Universidad de Sonora. Tilapia (*Oreochromis niloticus*) were donated by the Instituto de Acuacultura del Estado de Sonora. Fifty tilapias were randomly distributed in five 20 L aquaria, each filled with 18 L of fresh water, and the fish were acclimated for one week. The experimental design consisted of two treatments according to the sample method: whole gut content scraping (S) or gathered from the aquariumbottom (A). The scraping method will be considered as the control, which is the traditional method for gut microbiota sampling . The experimental conditions were constant aeration (dissolved oxygen ~6.5 mg/L), temperature of 25±0.5°C, and pH 7.7-7-8, and photoperiod 12:12. Fish had an average weight of 20.5 g and were fed three times a day with 4–5% of their body weight daily using a commercial diet containing 45% protein (Purina ®). The unconsumed feed and feces were siphoned to remove material from the previous feeding day. After acclimatization, the bioassay continued for a week, after which samples were collected after seven days.

Sample Collection

Two collection methods were used to retrieve fecal samples. The first consisted of a non-invasive method. To eliminate any organic matter, the aquariums were siphoned before sampling, and then aseptically collected fecal samples (A) from the aquarium-bottom. Sterile disposable plastic pipettes per aquarium were used to retrieve samples every 2 hours for 6 hours. After carefully removing the residual aquarium water by pipetting, the samples were placed in sterile tubes and stored at -80°C until DNA extraction. In the second method, after a 6-hour time-restricted feeding period, fish were ethically euthanized through anesthesia and the application of tricaine methanesulfonate (50 mg/L). The whole intestinal content was aseptically collected by the scraping (S) method from each fish, and samples were finally placed into sterile tubes and stored at -80°C. The study was conducted under the Guidelines for Ethical Conduct in the Care and Use of Nonhuman Animals in Research (2022), with the permission of the internal CIAD Ethics Committee (Registration: CONBIOÉTICA-26-CEI-001-20200122).

DNA Extraction and Sequencing

From each aquarium, three fish were randomly sampled using the scraping method, and three aquarium-bottom sites were randomly selected for direct water feces collection. Samples from the five aquaria were pooled and homogenized (FastPrep 5G, MP Biomedicals), and five replicates from the pool were obtained from each treatment to proceed with DNA extraction. DNA extraction from fecal samples was performed using a commercial Fast DNA™ spin kit for soil (Himachal Pradesh, INDIA) following the manufacturer's instructions.

The construction of libraries was performed following the 16S Metagenomic Sequencing Library guide with modifications. An initial amplicon PCR of the V9 variable region of the 18S rRNA gene was performed. The PCR primers with adapters were: 1391F 18S 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GTACACACCGCCCGTC and EukBrR 18S 5’GTCTCGTGGGCTCGGAGATGTGTATAAGAGACA GTGATCCTTCTGCAGGTTCACCTAC

The 24 µL PCR mixture contained 10 µL Platinum® PCR SuperMix (Invitrogen), 1 µL of each forward and reverse primer, 6 µL nuclease-free water, and 2 µL of template DNA. The PCR amplification conditions were an initial 94°C, 3-minute denaturation step, followed by 30 cycles of 94°C for 30 s, 57°C for 60 s, and 72 °C for 90 s before a final 10-minute extension at 72°C (Amaral-Zettler et al., 2009). The PCR products were visualized using agarose gel electrophoresis and quantified using the Qubit 3.0 fluorometer (Invitrogen). An indexing PCR

to add Nextera DNA indexes was used for sample multiplexing. The PCR products were cleaned up after each PCR protocol by using magnetic beads. Before pooling libraries, PCR products were normalized at 4 nM, then pooled by using 10 µL of each library. Libraries were diluted at 1 nM, denatured with 0.1 N NaOH, and combined with 5% (*v*/v) denatured 4 nM PhiX as a control. The sequencing was performed in an Illumina MiniSeq platform, using the MiniSeq Reagent Kit v2, yielding 2 × 150 bp paired-end reads. Raw reads were uploaded to NCBI, BioProject SRA accession: PRJNA1274963.

Data Processing

The initial raw demultiplexed sequence data were imported into QIIME2 version 2023.5 (Bolyen et al., 2019). The 859,466 reads were quality-filtered, denoised, and merged; chimeric reads were removed using DADA2. Reads were trimmed (1 to 149 bp) to remove low-quality regions. Additionally, the Amplicon Sequence Variants (ASVs) were summarized using the DADA2 algorithm (Callahan et al., 2016) and aligned and filtered with MAFFT v.7 (Katoh & Standley, 2013). Representative sequences were taxonomically assigned using classify-consensus-blast trained with a curated eukaryote-wide reference database, EUKARYOME, which compiles well-annotated, non-redundant, high-quality reads for the SSU marker subsets for high-accuracy taxonomic reference and chimera recognition (Tedersoo et al., 2024). After the data quality filter, a total of 802,784 reads were considered for further analysis. The diversity analysis was estimated with MicrobiomeAnalyst 2.0 (https://www.microbiomeanalyst.ca/). For alpha diversity estimation, Chao1, Shannon, Simpson, and Fisher indexes were used, and significant differences were assessed by performing one-way ANOVA ($P<0.05$), and results are shown as mean±SE (standard error) for each treatment group. Beta diversity was evaluated to assess the differences between microbial communities and visualized using PCoA (Principal Coordinate Analysis). Distance matrices were estimated based on Bray-Curtis, Jaccard, and weighted and unweighted phylogenetic Unifrac metrics. A statistical comparison was made between the composition of the microbial community by the permutational multivariate analysis of variance (PERMANOVA), and F-value, R-squared, and p-value were estimated for each beta diversity metric.

A heatmap was performed using Ward's clustering algorithm, in conjunction with the Euclidean distance measure, to group data points into clusters based on minimizing within-cluster variance. Random Forest (RF) analysis was applied to identify the most significant microbial features of the gut microeukaryotic community of our dataset, with 1000 estimators (trees). The RF algorithm is an ensemble machine learning model that combines multiple decision trees (Breiman, 2001; Chong et al., 2020; Dhariwal et al., 2017).

Results

Alpha diversity metrics revealed clear differences in the eukaryotic communities depending on the sample collection method. While the richness estimators (Chao1 and Fisher) did not show significant variation between methods ($p= 0.53$ and $p= 0.491$, respectively), diversity indices that account for evenness (Shannon and Simpson) showed significantly higher values in samples collected from the aquarium-bottom ($p= 0.024$ and $p= 0.044$, respectively), suggesting greater community uniformity in environmental samples (Figure 1).

Beta diversity analyses using both non-phylogenetic (Bray-Curtis, Jensen-Shannon) and phylogenetic (weighted and unweighted UniFrac) indices consistently indicated significant differences in the composition and structure of microeukaryotic communities between sample types (PERMANOVA, $P<0.05$) (Figure 2). These results confirm that the sampling method is a strong determinant of community configuration, even at broad taxonomic levels.

Taxonomic composition analysis at the phylum level further revealed divergent profiles between methods. Heatmap clustering showed that phyla such as Straminipila, Rhizaria, and Alveolata were enriched in samples obtained by intestinal scraping, likely representing gut-resident taxa (Figure 3). Similarly, the Random Forest classification identified Straminipila, Alveolata, and Rhizaria as the taxa contributing most strongly to distinguishing intestinal content from water-collected feces. Whereas Metazoa and other low-importance taxa (Euglenozoa, Choanoflagellozoa) appear more abundant in water samples, consistent with environmental contamination or post-defecation (Figure 4).

Discussion

Our results demonstrate that the sampling method used has a substantial impact on characterizing the fish gut microbiota, particularly affecting the structure and detection of eukaryotic communities. Samples obtained directly from the intestinal tract revealed a greater diversity of microeukaryotes and showed fewer environmental contaminants compared to fecal samples collected from the water, suggesting that direct intestinal sampling provides a more accurate representation of host-associated communities. These findings are consistent with other methodological insights that collectively emphasize the strong influence of sampling depth and method on observed community structure, such as the relative abundance of microorganisms or community composition (Ruiz, Torrecillas, et al., 2024; Tang et al., 2020; Thormar et al., 2024).

Intestinal contents and mucosa-adherent samples capture a richer, host-associated eukaryome, whereas water- or tank-collected feces often include transient

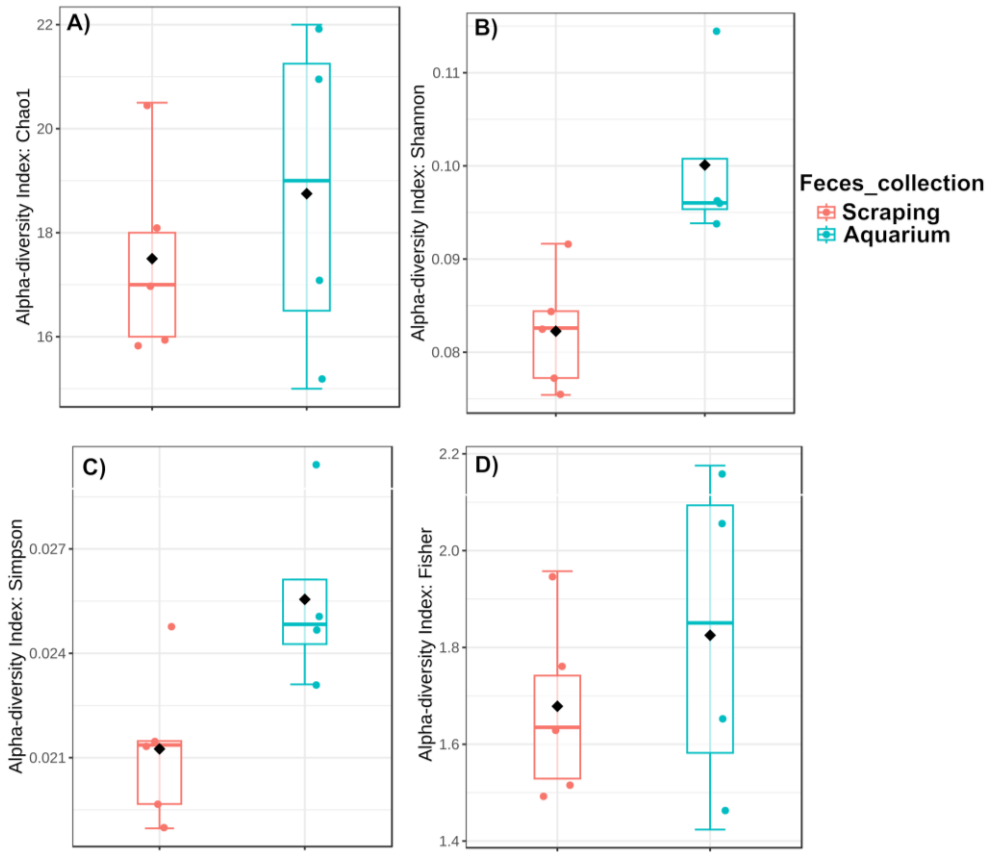

Figure 1. Alpha-Diversity of microeukaryotic communities using the scraping method (S) or gathered from the aquaria bottom (A). The Chao1 (A) (p= 0.53), Shannon (B) (p= 0.024). Simpson (C) (p= 0.044) and Fisher (D) (p= 0.491) indices were estimated. Data are shown as mean ± SE (standard error) for each treatment group.

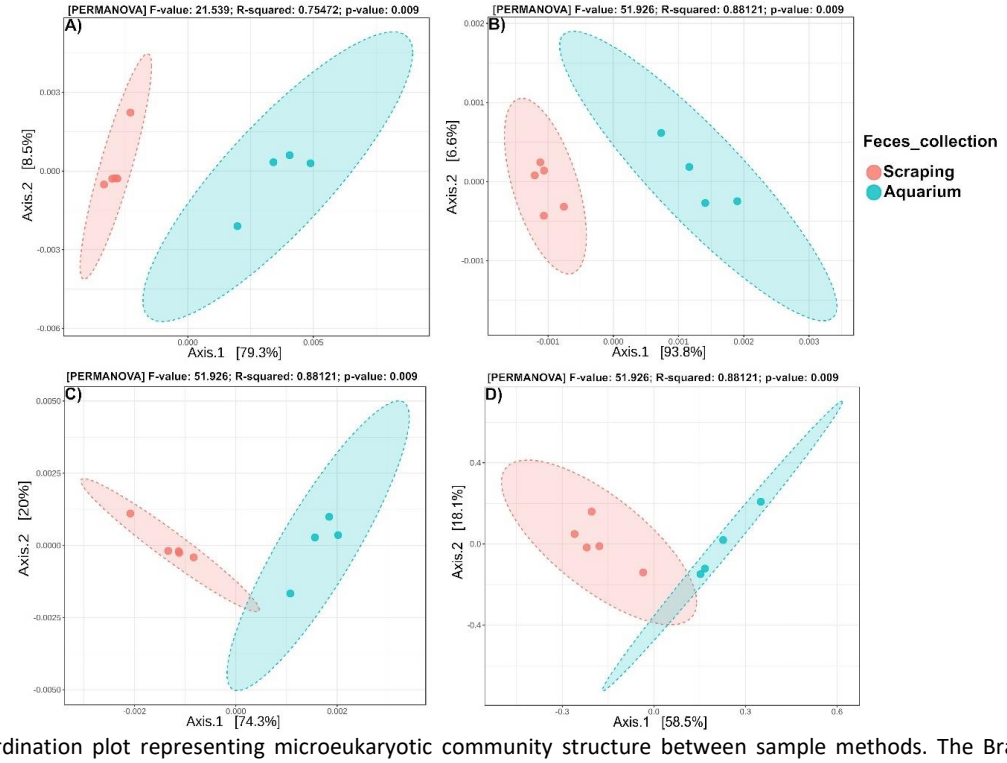

Figure 2. Ordination plot representing microeukaryotic community structure between sample methods. The Bray-Curtis (A), Jensen-Shannon (B), Weighted (C), and Unweighted Unifrac (D) distances were estimated. PERMANOVA was used to assess statistical differences: Bray-Curtis index (F-value: 21.539; R-squared: 0.75472; p-value: 0.009); Jensen-Shannon (F-value: 51.926; R-squared: 0.88121; p-value: 0.009); Weighted Unifrac (F-value: 15.865; R-squared: 0.69386; p-value: 0.009); Unweighted Unifrac (F-value: 7.5525; R-squared: 0.51898; p-value: 0.009).

environmental taxa that can inflate diversity estimates and obscure host-associated communities (Guirado-Flores et al., 2025). Water-collected fecal samples often reflect environmental taxa rather than true gut colonizers, potentially overestimating transient organisms and underrepresenting mucosa-associated or low-abundance symbionts, which can bias interpretations of host-microeukaryote interactions (Anslan et al., 2019; Nolorbe-Payahua et al., 2020).

Notably, intestinal samples contained filamentous fungi such as *Fusarium* and *Cladosporium*, along with protozoa potentially involved in commensal or symbiotic interactions. In contrast, fecal samples collected from the environment were dominated by cosmopolitan taxa such as *Candida tropicalis*, which are commonly associated with aquatic substrates. These findings are consistent with observations by Ghanbari et al. (2015), who reported that in *Lates calcarifer*, intestinal samples harbored more stable bacterial communities, while feces collected from the water were enriched with environmental taxa like *Pseudomonas* and *Aeromonas*. Such patterns suggest post-excretion colonization and highlight the limited utility of ambient fecal samples for studying host-associated microbiota.

Although empirical data on eukaryotic gut communities in fish remain scarce, similar ecological principles apply. Once expelled into the aquatic environment, fecal matter is exposed to rapid degradation, oxidative stress, and colonization by opportunistic microorganisms. This can distort the original community structure and reduce the resolution of downstream analyses. As Ni et al. (2014) emphasized, the gut of fish functions as a selective microenvironment shaped by pH, immune regulation, and nutrient gradients, which together support the establishment of specialized microbial consortia. The discrepancy between environmental and intestinal samples thus reflects the fundamental differences between transient and resident communities.

A limitation of this study is the small sample size, which may reduce statistical power and reduce the complete outlook in the low-abundance microeukaryotic taxa. Future studies with larger sample sizes are needed to strengthen ecological and functional interpretations. Moreover, we observed greater consistency in eukaryotic community profiles among intestinal replicates, while fecal samples exhibited higher inter-individual variability. This reinforces the importance of sampling directly from the gut for studies aiming to detect fine-scale patterns or subtle taxonomic groups, such as fungi and protozoa.

Finally, these findings underscore the methodological implications of sampling strategy in gut microbiota research. While non-lethal fecal sampling from the water may offer logistical convenience and a useful proxy for gut microbial communities, particularly for longitudinal studies, it compromises the ecological accuracy of community assessments, particularly when investigating mucosa-adherent or low-abundance taxa. Direct intestinal sampling, though more invasive, should be considered the preferred approach when the goal is to capture the composition and structure of the true resident microbiota (Andreas Härer & Diana J. Rennison, 2023).

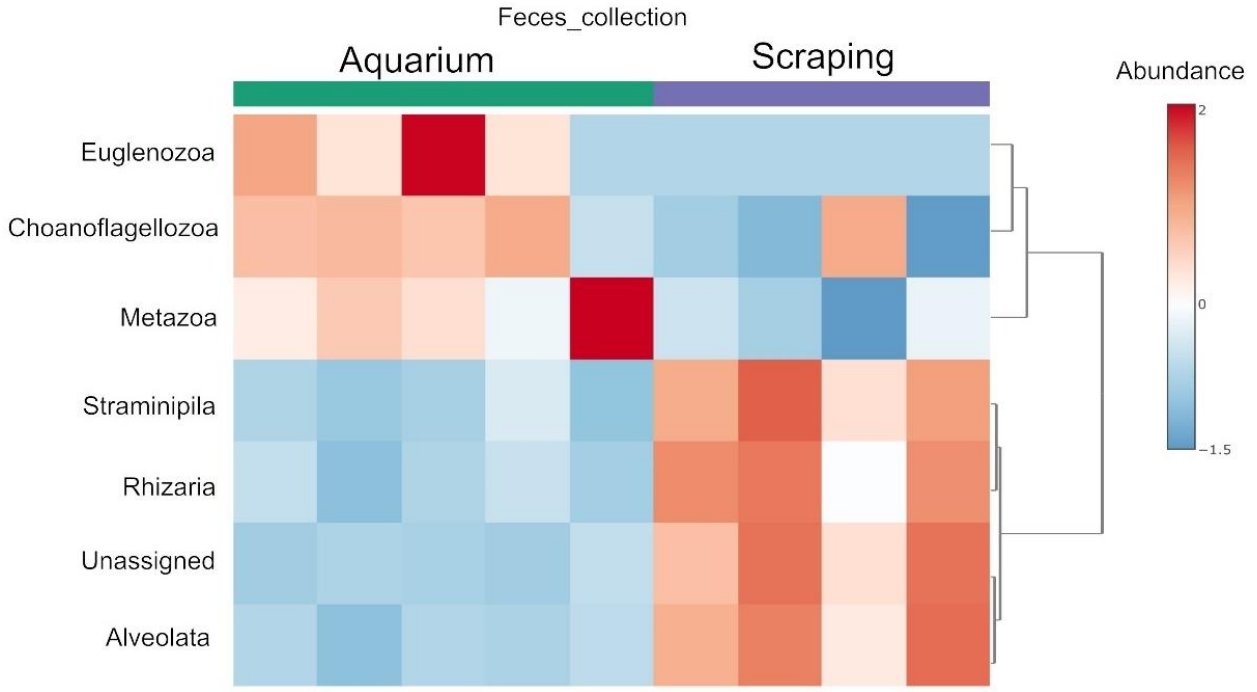

Figure 3. Heatmap analysis of the tilapia gut microbiota at the phylum level related to its sample method. A clustering algorithm (Ward), in conjunction with the Euclidean distance, was used to construct the heatmap analysis.

Conclusions

This study demonstrates that the sampling method exerts a substantial influence on the diversity and composition of microeukaryotic communities in the fish intestine. While bacterial profiles may tolerate broader methodological variation, eukaryotic communities appear more sensitive to whether samples are collected directly from the host or the surrounding environment. Research approaches should carefully consider and report their sampling protocols, especially when investigating the functional roles of gut microbiota.

Future studies should establish standardized protocols that clearly differentiate host-associated from environmental communities, enabling more consistent cross-study comparisons. Integrating multi-omic approaches could provide deeper insights into the ecological and functional roles of specific microeukaryotes within the gut. Experimental validation of host–microeukaryote interactions, along with controlled manipulative studies under both laboratory and natural conditions, will be essential to understand how sampling strategies shape our interpretation of intestinal symbioses.

Overall, analytical objectives, whether the aim is to characterize host-associated eukaryotes or environmental exposure, and sampling methods, accordingly, must be defined. When feasible, invasive approaches may provide more accurate insights into endogenous gut communities, particularly for sensitive or obligate symbionts.

Ethical Statement

The study was conducted under the Guidelines for Ethical Conduct in the Care and Use of Nonhuman Animals in Research (2022), with the permission of the internal CIAD Ethics Committee (Registration: CONBIOÉTICA-26-CEI-001-20200122). All sampling was performed under tricaine methanesulfonate anesthesia, and all efforts were made to minimize suffering.

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Author Contribution

Conceptualization: EGV, JSOGF, MMP, Data Curation: JSOGF, EGV, Formal Analysis: EGV, Investigation: JSOGF, Methodology: JSOGF, EGV, Project Administration: MMP, FVA, EGV, Resources: MMP, FVA, LRMC, Supervision: EGV, Validation: MMP, RAVG, Visualization: EGV, RAVG, Writing – Original Draft Preparation: EGV, MMP, RAVG, Writing – Review & Editing: EGV, MMP, RAVG.

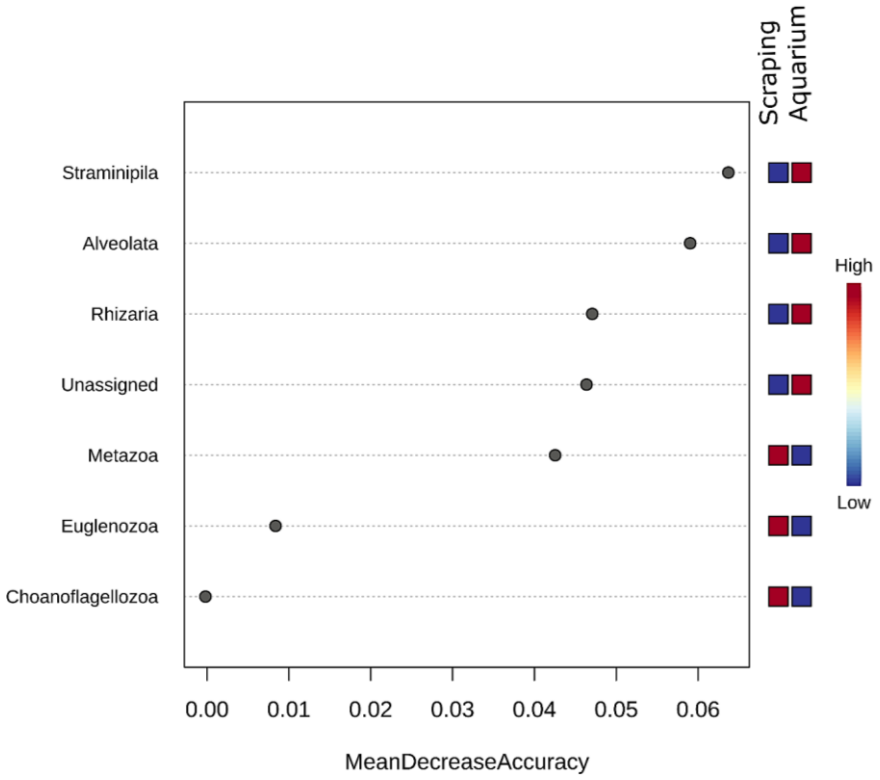

Figure 4. Importance of eukaryotic taxa in classifying samples from scraping and water based on Random Forest analysis (1000 trees). The Mean Decrease Accuracy represents the contribution of each taxonomic group to model accuracy, which is ranked by its contribution to classification accuracy. Taxa with higher values indicate greater relevance in distinguishing between sample methods. The side color scale reflects the relative abundance of each group, with red indicating higher abundance and blue lower abundance across sample methods. The Straminipila, Alveolata, and Rhizaria as the main taxa that contribute to distinguishing intestinal content (scraping) from water-collected feces.

Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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