DETECTION OF BIOLOGICAL RAW MATERIALS AND POTENTIAL EUKARYOTIC PATHOGENS IN FEED INGREDIENTS THROUGH HIGH-THROUGHPUT SEQUENCING

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Abstract. Feed quality is crucial for both animal health and human food safety. However, traditional testing methods for analyzing the raw components of feed ingredients are cumbersome and often target only a single detection parameter. To assess the effectiveness of high-throughput sequencing technology in detecting both feed raw materials and potential eukaryotic pathogens, this study evaluated the eukaryotic composition of raw materials using three pairs of eukaryotic primers. Our results showed significant variations in species annotation among the sequences obtained from different primers. Specifically, the 18S rRNA gene primer pairs (Uni18S and Uni18SR, and TAReuk454FWD1 and TAReukREV3) were able to detect a wide range of species and particularly effective at identifying fungi, whereas the 12S rRNA gene primer pair (AcMDB07-F and AcMDB07-R) provided more detailed identifications of multicellular ingredient sources. These findings suggest that using multiple pairs of primers can provide a comprehensive analysis of the true composition of feed ingredients. The substantial differences in species abundance observed in our study highlight the importance of careful selection of primers for species detecting, which can provide crucial data for the development of future feed safety evaluation methods.

Keywords: *feed quality control, feed industry, 18S rRNA, 12S rRNA, new-generation sequencing, primer*

Introduction

Livestock account for nearly 40% of total agricultural production in developed countries and 20% in developing countries, supporting the livelihoods of at least 1.3 billion people worldwide (FAO, 2023). Livestock provide 35% of the world's dietary protein (Wu et al., 2014), and 86% of livestock feed is made from resources inedible to humans, consuming about one-third of total global cereal production (Mottet et al., 2017). Grains and grain-based products are the most common components of human and animal diets and are also well-suited raw materials for mold growth (Nelson, 1993; Mohapatra et al., 2017; Poutanen et al., 2022; Kassa et al., 2023). Fungal spoilage of products manufactured by the food and beverage industry imposes significant annual global revenue losses (Rico-Munoz et al., 2019). The absence of visible mold on microscopic examination does not necessarily indicate that food or feed is free of antitoxins (Basak et al., 2021; Dagnas and Membré, 2013).

The use of cheap plant proteins to replace more expensive animal-source proteins in animal feed is a common practice by some feed manufacturers to reduce costs. However, this practice significantly increases the risk of toxin contamination in animal feed (Kim et al., 2019; Parisi et al., 2020; Gurikar et al., 2023; Pexas et al., 2023; Thornton et al., 2023). This poses a serious threat to the feed supply chain, animal health, and human food safety (Bryden, 2012; Xiao et al., 2023; Medeiros et al., 2024). The composition of the gastrointestinal microbiota of animals varies depending on their dietary behavior and feed components, leading to a high incidence of various gastrointestinal diseases (Ni et al., 2014; Hou et al., 2022; Li et al., 2023; Barathan et al., 2024; Liu et al., 2024). Therefore, controlling the nutrition in animal feed is crucial in preventing many diseases during feeding (Cerf, 2021; Abu Hafsa et al., 2022; Tuomilehto, 2022; Mamphogoro et al., 2024; Predescu et al., 2024). The adulteration of feedstuffs can cause a range of metabolic diseases and immune problems in fed animals (Čolović et al., 2019; Momtaz et al., 2023; Anagaw et al., 2024). Furthermore, contamination during production, transportation, storage, and feeding of feedstuffs can result in massive animal deaths and pose serious public health challenges (Ibarra et al., 2018; Meulenbelt, 2018; Pakdel et al., 2023). As animal husbandry becomes increasingly intensified, the importance of maintaining the health of the animals being fed becomes more apparent (Ducrot et al., 2024). While traditional and emerging feed quality inspection techniques are widely used, these methods are often influenced by subjective factors, complex sample processing, and instrumentation, which may hinder a comprehensive and objective assessment of the real components of feed (Cheli et al., 2012; van der Poel et al., 2020; Hassoun et al., 2020; Artavia et al., 2021).

Currently, high-throughput sequencing technology has become increasingly popular in biological research and medical fields due to its speed, low cost, high accuracy, and low sample requirements compared to first-generation sequencing (Ni et al., 2014, 2021; Wu et al., 2022; Fu et al., 2023). This technology also offers new opportunities for studying the raw components of feed (Imanian et al., 2022). Amplicon sequencing, which is based on ribosomal RNA genes, is a significant technical method for analyzing eukaryotic composition (Choi and Park, 2020; Xu et al., 2020; Bukin et al., 2023; Wu et al., 2023). However, there is currently a lack of literature on relevant feed quality control technologies. Therefore, in addressing issues related to feed component sources, it is possible to overcome the limitations of current quality control methods by utilizing high-throughput sequencing technology and polymerase chain reaction (PCR)-based quantitative methods. These methods can identify species and analyze the composition of communities in feed samples, effectively detecting quality issues such as mold, adulteration, and component source fraud.

Primer selection is crucial for accurately taxonomically characterizing biomes, as no single primer pair has been found to distinguish all eukaryotes simultaneously (Vaulot et al., 2022; Zheng et al., 2022; Bukin et al., 2023). The primer pairs must be designed to target specific ribosomal RNA genes, have an appropriate amplicon size, and minimize mismatches to amplify the target population without bias. This is important because traditional culture methods do not capture the full diversity of organisms' present (Hugerth et al., 2014). However, the use of different primers and databases may result in the failure to detect certain toxin-producing fungi or poor-quality components, leading to inaccurate analytical results. Therefore, the selection of appropriate primer pairs and optimization of sequence reference databases are crucial for the successful application of high-throughput sequencing technology in feed quality control. The objective of this study was to use three primer pairs to analyze the species composition of different feed formulations using high-throughput sequencing technology, compare their ability to detect eukaryotes, particularly eukaryotic pathogens, and identify the most suitable primers for quality control analysis of feed components.

Materials and methods

Experimental design and sample sources

Three commonly used primer pairs were utilized in this study: AcMDB07-F (5′ – GCC TAT ATA CCG CCG TCG – 3′) and AcMDB07-R (5′ – GTA CAC TTA CCA TGT TAC GAC TT – 3′) for the fish 12S rRNA gene (Shu et al., 2021; Xiong et al., 2022), Uni18S (5′ – AGG GCA AKY CTG GTG CCA GC – 3′) and Uni18SR (5′ – GRC GGT ATC TRA TCG YCT T – 3′) for the eukaryotic hypervariable V4 region of the 18S rRNA gene (Zhan et al., 2013; Flynn et al., 2015), and TAReuk454FWD1 (5′ – CCA GCA SCY GCG GTA ATT CC – 3′) and TAReukREV3 (5′ – ACT TTC GTT CTT GAT YRA – 3′) for the eukaryotic hypervariable V4 region of the 18S rRNA gene (Lejzerowicz et al., 2015; Fonseca et al., 2022). These primer pairs were selected to analyze the eukaryotic species composition in the samples being studied. The reasons for choosing these specific primer pairs are twofold: firstly, the composition of fish meal in aquatic feed is crucial for feed nutrition, and we aimed to obtain information on the fish species present in the samples using the AcMDB07-F/AcMDB07-R primer pair; secondly, we wanted to compare the results obtained using the Uni18S/Uni18SR and TAReuk454FWD1/TAReukREV3 primer pairs, and their effectiveness in analyzing fungi, particularly molds.

To validate the effectiveness of these primer pairs in identifying species, we conducted an analysis on six samples of feed and feed raw materials for aquaculture. These samples included S1, which was a commercial *Channa argus* feed (Jieda, Foshan, China), S2, which was a commercial *Ctenopharyngodon idella* feed (Haid, Guangzhou, China), and S3 to S6, which were feed raw materials mixed with chicken (Daynew, Foshan, China), tilapia (Daynew, Foshan, China), and Peruvian fish (TASA, Peruvian) meals (*Table 1*). The commercial feeds (S1, and S2) were randomly weighted approximate 5 g, crushed, and mixed, and then taken 0.3 g for DNA extraction. The feed raw materials were mixed to approximate 5 g according to *Table 1,* and then taken 0.3 g for DNA extraction.

Table 1. *Feed raw material information in this study. Chicken and tilapia meals were obtained from Daynew Co. Ltd. (Foshan, China), and Peruvian fish meal was purchased from TASA (Peruvian)*

Sample name	Chicken meal	Tilapia meal	Peruvian fish meals
S3	15	59	20
S4	47	28	89
S5	83	46	30
S6	15	55	66

DNA extraction and sequencing

DNA was extracted from the feeds or feed components using a FastDNA spin kit for soil (MP, California, USA). The extracted DNA was detected using 1.8% agarose gel electrophoresis. The eukaryotic hypervariable V4 region of the 18S rRNA gene was amplified using the primer pairs Uni18S and Uni18SR, and TAReuk454FWD1 and TAReukREV3, while the fish 12S rRNA gene was amplified using the primer pair AcMDB07-F and AcMDB07-R, as previously described (Zhan et al., 2013; Flynn et al., 2015; Lejzerowicz et al., 2015; Shu et al., 2021; Xiong et al., 2022). Briefly, PCRs were performed in duplicate with a 25-µL reaction mix containing 1 × PCR buffer, 0.25 U of EasyTaq DNA polymerase (TranGen Biotech, Beijing, China), 0.2 mM of each high-purity deoxynucleoside triphosphate (TranGen Biotech, Beijing, China), 1.0 µM of each primer and 10 ng of DNA. The thermal cycling procedure consisted of an initial pre-denaturation step at 94°C for 10 min, followed by 30 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 10 min. After amplification, the PCR products were electrophoresed using a 1.8% agarose gel, and the correct band was extracted and purified using an AxyPrep DNA gel extraction kit (AxyGen, Suzhou, China). The purified DNA was then quantified using a Nanodrop 2000 spectrophotometer (ThermoFisher Scientific, USA). All purified amplicons were pooled together with an equal molar amount from each sample and sequenced using an HiSeq system (Illumina, USA) at Guangdong Meilikang Bio-Science, Ltd. (Foshan, China).

Data analysis

The raw reads were merged using FLASH 1.2.8 (Magoč and Salzberg, 2011). All merged tags were trimmed and assigned to each sample based to their barcode sequences, with a maximum of o mismatch allowed using QIIME 1.9.0 (Caporaso et al., 2010). Subsequently, the low-quality tags (length < 300 bp, containing ambiguous bases, or with an average base quality score < 30) were removed to obtain high-quality tags. The Uchime algorithm was then used to identify and remove any chimera sequences from the high-quality tags, before clustering them into operational taxonomic units (OTUs) with 97% identity using UPARSE (Edgar, 2013).

To accurately annotate species, we utilized a multi-step approach. Firstly, we identified and aligned the representative sequences of the top 50 OTU from all samples for each primer pair using BLASTn against the eukaryotic sequence dataset in the NCBI experimental database. Any OTUs that showed the highest similarity to known sequences were recorded, along with their scientific names and accession numbers. Non-target sequences were excluded from this process. OTUs with low abundance were categorized as “Other”. To compare the taxonomic composition at different classification levels, we cross-referenced the scientific names retrieved from NCBI with the NCBI taxonomy database. We assigned reliable taxonomic annotations at standard classification ranks, including domain, phylum, class, order, family, genus, and species. Any unclassified taxa were labeled as “Unidentified”. Finally, we merged identical species counts and calculated the relative abundance proportions per sample. To visually represent the phylum- and species-level compositions, we generated stacked bar plots using the ggplot2 package (Wickham, 2016) in R 4.2.3 (R Core Team, 2021).

To evaluate the differential detection capabilities of different primers, we utilized the eulerr package (Larsson et al., 2024) in R to perform set operations on the phylum- and species-level annotations. This allowed us to visualize the overlaps through Venn diagram. Furthermore, we used the pheatmap package (Kolde, 2018) in R to create heatmaps of eukaryotic species abundances detected in both feed and feed ingredients across the three primer pairs. These heatmaps displayed the relative abundance and the log-transformed relative abundance of each species.

Results

A total of 61,977 high-quality sequences were obtained from the six different feeds using three primer pairs that amplify the 18S rRNA V4 region and 12S rRNA fragments. After applying a 97% sequence identity threshold, 897 OTUs were identified. Sequences, except for those with undefined class names and unverified OTUs, were classified into 15 phyla (*Fig. 1A*), and a total of 80 species were detected across all samples (*Fig. 1B*). As expected, the use of different primer pairs resulted in distinct eukaryotic community structures from the same feeds (*Fig. 1; Appendix 1*).

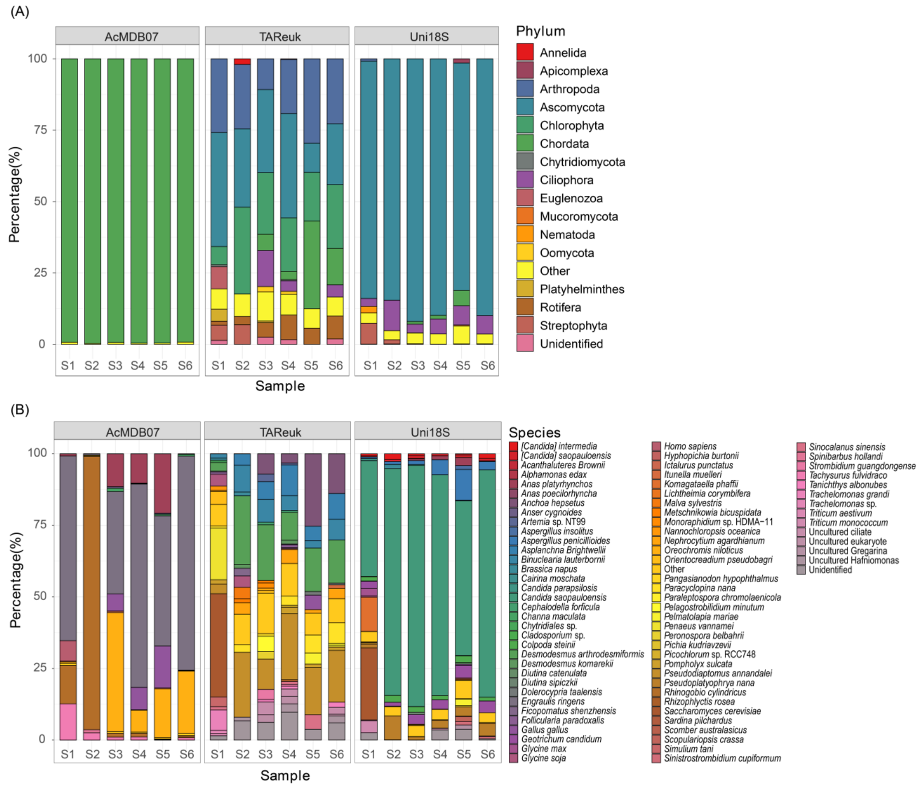

Figure 1. *Phylum (A) and species (B) compositions in the feed samples analyzed using three different primer pairs*

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The two primer pairs, Uni18S and TAReuk, amplify the 18S rRNA V4 hypervariable region, revealing a wide range of eukaryotic diversity (*Fig. 1*). The TAReuk primer pair covered a diverse range of taxa including Arthropoda, Ascomycota, Chlorophyta, Chordata, Ciliophora, Euglenozoa, Oomycota, Rotifera, and Streptophyla, whereas the Uni18S primer pair covered Apicomplexa, Ascomycota, Chordata, Euglenozoa, Nematoda, and Rotifera. Furthermore, the Uni18S primer pair showed a better detection effect on fungi (*Fig. 1A*). At the species level, there was no significant difference in the species richness detected by the two primer pairs ($P > 0.05$; *Appendix 1*), whereas the TAReuk primer pair detected a higher Shannon index ($P < 0.05$; *Appendix 1*). The dominant species (their relative abundance > 1%) detected by the TAReuk primer pair in sample S1 were *Saccharomyces cerevisiae*, *Penaeus vannamei*, *Trachelomonas grandi*, *Orientocreadium pseudobagri*, *Glycine soja*, *Pseudodiaptomus annandalei*, *Simulium tani*, *Desmodesmus arthrodesmiformis*, *Binuclearia lauterbornii*, *Pompholyx sulcata*, *Nannochloropsis oceanica*, *Sinocalanus sinensis*, and *Trachelomonas sp*. (*Fig. 2*). In sample S2, the dominant species detected by the TAReuk primer pair were *D. arthrodesmiformis*, *P. annandalei*, *B. lauterbornii*, *G. soja*, *Nephrocytium agardhianum*, *Asplanchna brightwellii*, *Malva sylvestris*, *Paracyclopina nana*, *Ficopomatus shenzhensis*, *Brassica napus*, *Desmodesmus komarekii*, and *Metschnikowia bicuspidata* (*Fig. 2*). In samples S3-S6, the TAReuk primer pair detected *P. annandalei*, *D. arthrodesmiformis*, *Anchoa hepsetus*, *A. brightwellii*, *B. lauterbornii*, *P. nana*, *N. agardhianum*, *S. sinensis*, *Pelagostrobilidium minutum*, *Gallus gallus*, *P. sulcata*, *Pelmatolapia mariae*, *Strombidium guangdongense*, *Tanichthys albonubes*, *Artemia sp. NT99*, *Peronospora belbahrii*, *Dolerocypria taalensis*, *Monoraphidium sp. HDMA-11*, *Itunella muelleri*, and *D. komarekii* were detected as dominant species (*Fig. 2*). These results indicated that the TAReuk primer pair is effective in identifying fungi, plants, and animals in feeds. However, *Candida saopauloensis*, *S. cerevisiae*, *Komagataella phaffii*, *Triticum monococcum*, *Glycine max*, *Geotrichum candidum*, *Colpoda steinii*, and *Pseudoplatyophrya nana* were detected as dominant species from S1 using the Uni18S primer pair (*Fig. 3*). *C. saopauloensis*, *P. nana*, *C. steinii*, *Candida intermedia*, *B. napus*, and *G. candidum* were detected as dominant species from S2 (*Fig. 3*). *C. saopauloensis*, *P. nana*, *C. steinii*, *C. intermedia*, *G. candidum*, *Aspergillus penicillioides*, *Aspergillus insolitus*, *Anas platyrhynchos*, *P. mariae*, *Sinistrostrombidium cupiformum*, and *Scopulariopsis crassa* were detected as dominant species from S3-S6 (*Fig. 3*). Although the Uni18S primer pair exhibited stronger amplification effect on fungi than TAReuk, its over-amplification on *C. saopauloensis* limited its identification effect on other species, especially vertebrates.

The AcMDB07 primer pair, which amplifies the 12S rRNA gene, only detected Chordata (*Fig. 1A*). Engraulis ringens, Rhinogobio cylindricus, Tachysurus fulvidraco, Homo sapiens, A. platyrhynchos, Oreochromis niloticus, and Ictalurus punctatus were detected from the S1 using the primer pair, whereas R. cylindricus, T. fulvidraco, Spinibarbus hollandi, E. ringens, and H. sapiens were detected from the S2 (*Fig. 4; Appendix 2*). E. ringens, O. niloticus, A. platyrhynchos, G. gallus, R. cylindricus, T. fulvidraco, Channa maculata, I. punctatus, Anser cygnoides, Pangasianodon hypophthalmus, Acanthaluteres Brownii, H. sapiens, Cairina moschata, Scomber australasicus, Sardina pilchardus, and Anas poecilorhyncha were detected from the S3-S6 (*Fig. 4; Appendix 2*). These results indicated that the AcMDB07 primer pair accurately identified the ingredients of chicken, tilapia, and Peruvian fish meals.

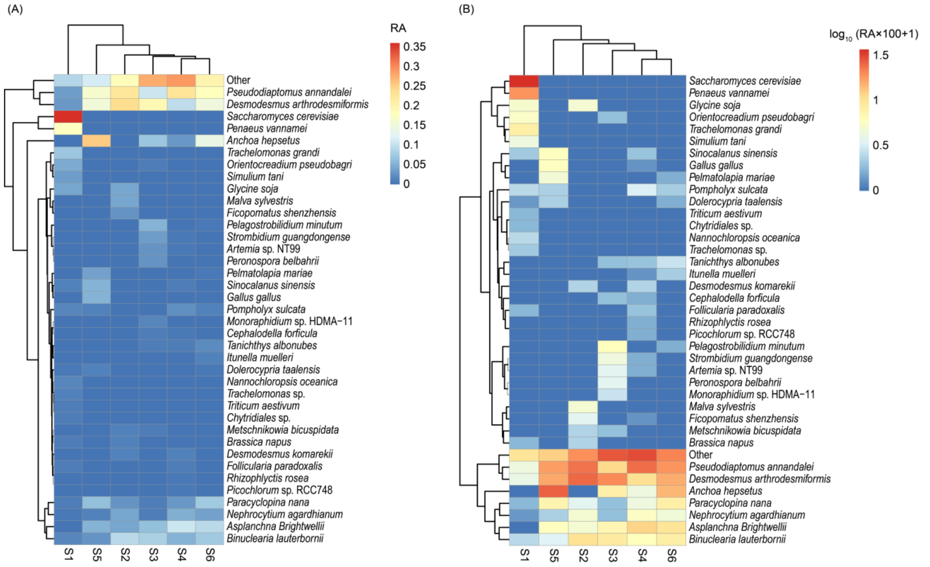

Figure 2. *Species composition of eukaryotes in the feed and feed raw materials detected using primer pair TAREuk. (A) Relative abundances; (B) Logarithmic transformation of the relative abundances*

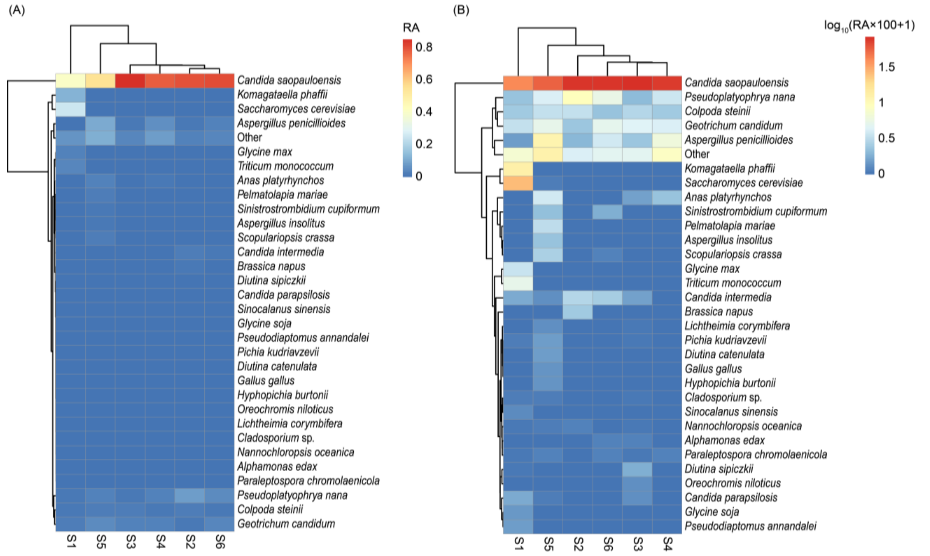

Figure 3. *Species composition of eukaryotes in the feed and feed raw materials detected using primer pair Uni18S. (A) Relative abundances; (B) Logarithmic transformation of the relative abundances*

Discussion

Since the publication of the first large-scale phylogenetic studies in the animal kingdom based on 18S rRNA sequences by Field et al. (1988), this gene has been recognized as a prime candidate for reconstructing phylogenetic trees. Currently, target gene-based amplicon sequencing is the most widely used high-throughput sequencing application in bioecology, overcoming the limitations of traditional culture methods that cannot access the entire compositional diversity of organisms. Our evaluation of the effectiveness of different primer assays based on feed samples confirmed the ability of two previously published primer pairs for 18S rRNA (Uni18S and TAREuk) to detect eukaryotes with high coverage (Stoeck et al., 2010; Zhan et al., 2013). However, our analysis also found that most species detected by TAREuk primers were invertebrates, some algae, and rare fungus-like protists in the samples. In contrast, an overwhelming proportion (86.21%) of OTUs detected by Uni18S primers were fungi. Additionally, considering the shortcomings of phylogenetic analyses based on individual ribosomal RNA genes, the detection of fish species in fishmeal, an important source of feed protein for animal-derived feeds, was enhanced by a mitochondrial 12S rRNA primer (AcMDB07) developed and validated by Bylemans et al. (2018). This primer compensates for the poor fish-specific amplification of the other two primer pairs.

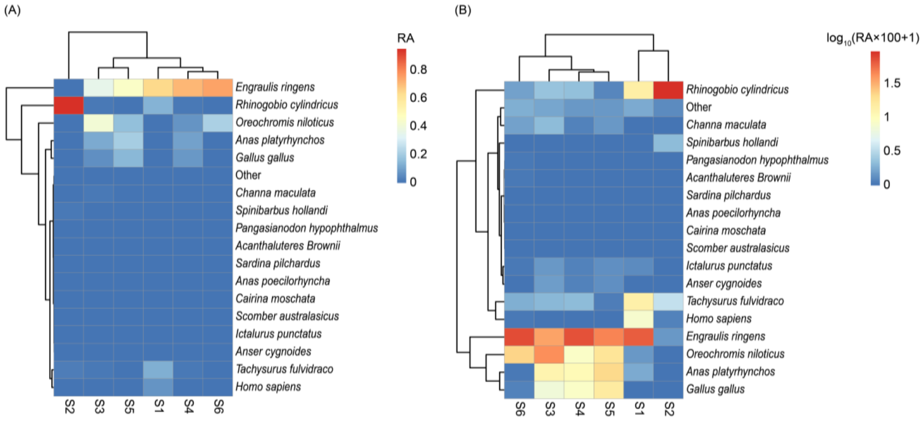

Figure 4. *Species composition of eukaryotes in the feed and feed raw materials detected using primer pair AcMDB07. (A) Relative abundances; (B) Logarithmic transformation of the relative abundances*

Primers designed to amplify different rRNA genes or different conserved regions within highly variable regions of the same rRNA gene can significantly affect the number of species detected and the proportional abundance data. However, careful selection of primers and the use of multiple primer pairs for analysis can reduce the impact of similar amplification biases, resulting in more comprehensive information on the detected species. For instance, although TAReuk454FWD1 and TAReukREV3 were previously reported to be used for amplifying fungal ITS rDNA (Sui et al., 2022), our results confirmed that the primer pair also amplified eukaryotic 18S rDNA sequences. Similarly, although the primer pair AcMDB07-F and AcMDB07-R was originally designed for amplifying the 12S rRNA gene in fish, our results showed that it could also amplify 12S rRNA gene sequences from other organisms such as birds and human. However, it should be noted that although all three primer pairs amplified human gene sequences, these sequences were probably unintentionally contaminated during the processing of feed or raw materials, rather than intentionally added.

The composition of feed varies depending on the crop, feeding objectives, and growth stage. It typically includes energy components, crude protein, crude fiber, crude fat, inorganic salts and minerals, vitamins, amino acids, sugars, and various types of feed additives (Feye et al., 2020). The nutrient content is primarily influenced by the biological raw materials used in the feed. For instance, post-weaning nutritional diarrhea (PWND) in piglets is often caused by dietary changes before and after weaning, which can lead to disruptions in the intestinal microbiota. The percentage of dietary proteins, fibers, and resistant starch, as well as the balance of electrolytes, can affect fermentation products and alter the composition of the gastrointestinal microbiota, ultimately resulting in PWND (Gao et al., 2019). Feeding experiments with varying levels of crude protein in feeds have revealed that different crude protein levels can impact the expression of aquaporin in the animal intestines, which can affect the balance of intestinal water absorption and secretion in piglets (Ren et al., 2023). In addition to common gastrointestinal diseases, unbalanced feed nutrition can also affect growth and reproduction, increase the risk of metabolic diseases and immune problems, and make animals more susceptible to infections and diseases. Animal-derived raw materials, such as fishmeal and meat and bone meal, are typically used as high-quality sources of protein and amino acids in finished feeds. However, due to rising prices, these materials are sometimes replaced with low-quality alternatives, such as processed feather meal, blood meal, and leather meal. Traditional testing methods are often unable to accurately detect the true composition of these adulterated materials, which can have negative impacts on the health and production performance of animals and harm the interests of aquaculture enterprises. In this study, the AcMDB07 primer pair was successfully used to detect chicken, tilapia, anchovy, sardine, and other fish species in our detected feed raw materials.

In addition to animal-derived ingredients, plants such as soybeans, soybean meal, and wheat are also commonly used in animal feed. These oil-rich grains not only serve as primary ingredients, but they are also highly susceptible to mold growth and reproduction. Mycotoxins are secondary metabolites produced by molds, and some of the most prevalent types found in feed include aflatoxins, fumonisins, ochratoxins, dinoflagellates, and zearalenones (Santos Pereira et al., 2019). These harmful substances can be present in various agricultural and food products and their levels can be influenced by factors such as moisture content, moisture activity, relative humidity, temperature, and pH (Sforza et al., 2006). When mold growth occurs in feed, it can have negative effects on animal health and productivity, including decreased daily weight gain, reduced egg or milk production, increased susceptibility to diseases, and decreased feed conversion efficiency. In severe cases, it can even lead to the development of tumors and malformations (Alshannaq and Yu, 2017). Safety concerns during feed production include the practice of mixing raw materials from different batches, which can create a new matrix with a completely different risk profile. Since mycotoxins are stable compounds, they are not destroyed during storage, milling, or high-temperature feed production. Therefore, it is crucial for feed manufacturers to prioritize contamination prevention over cost savings when selecting ingredients. Currently, mycotoxin detection methods primarily include chromatographic techniques such as high-performance liquid chromatography, gas chromatography, and liquid chromatography-mass spectrometry; immunological methods such as enzyme-linked immunosorbent assay and immunochromatographic paper; biosensors, including optical and electrochemical sensors; and molecularly imprinted technologies like molecularly imprinted polymers (Janik et al., 2021). However, most of these assays rely on specialized personnel and require high levels of sample handling. As living standards have increased, consumers have become more conscious of the nutritional quality of farmed products. More evidence is emerging about the potential role of feeds and ingredients as disease vectors (Stewart et al., 2020). If feeds contain deteriorated and harmful ingredients, feeding them to animals can harm their health and affect human health if residues are consumed (Paulk et al., 2024). We detected some fungi, such as

Candida spp. and *Aspergillus* spp., using two-pair amplification of the V4 region of the 18S rRNA gene, but no typical pathogenic strains were found. Fungi are commonly found as biological contaminants in the human environment, posing a serious threat to public health. *Candida* spp., particularly *Candida albicans*, is a major fungal pathogen in humans. It is present as a commensal in the oral, intestinal, or reproductive tracts of most human or animal hosts (Khan and Karuppayil, 2012). This fungus is highly susceptible to infection and transmission in immunocompromised individuals, making fungal diseases more common in captive animals (d'Enfert et al., 2021; Garcia-Bustos et al., 2024). However, our study did not identify any common eukaryotic pathogenic microorganisms. This may be due to the limited number of samples used in the study. Additionally, the choice of eukaryotic databases can greatly influence the results of species annotation. The current mainstream eukaryotic databases, such as the SILVA database, still have issues with a wide range of evolutionary clades, complex taxonomic criteria, and missing taxonomic levels of some species. This can make it difficult to accurately analyze the taxonomic composition of communities in environmental samples. Despite these limitations, this method can still be used to detect pathogenic microorganisms in feedstuffs. It breaks through the constraints of traditional quality control methods and can be utilized for preventing or detecting fungal diseases in animals.

Discussion

Our study highlights the significant impact that the choice of primers can have on the results of eukaryotic analysis of feed ingredients. Among the selected primer pairs, the Uni18S primer pair is the best choice for detecting fungi in feed, offering the highest detection ability and amplification efficiency. The TAREuk primers can detect a broader range of species, providing more comprehensive coverage. Although the AcMDB07 primer can only detect chordates in the sample, it can more accurately identify the true source of feed protein and can serve as a verification method for feed adulteration. We also discovered during the species annotation process that the confusion and gaps in annotation information in the current mainstream eukaryotic sequence reference libraries remain significant obstacles to the practical application of eukaryotic high-throughput sequencing technology. Therefore, these issues should be addressed promptly. Through careful selection of primer pairs, the development of high-quality eukaryotic reference databases, and advanced bioinformatics processing tools, eukaryotic high-throughput sequencing technology will become an increasingly reliable tool for analyzing the true components of feed ingredients.

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REFERENCES

[1] Abu Hafsa, S. H., Hassan, A. A., Elghandour, M. M., Barbabosa-Pliego, A., Mellado, M., Salem, A. Z. (2022): Dietary anti-nutritional factors and their roles in livestock nutrition. – Sustainable Agriculture Reviews 57: 127-142.

[2] Alshannaq, A., Yu, J.-H. (2017): Occurrence, toxicity, and analysis of major mycotoxins in food. – International Journal of Environmental Research and Public Health 14: 632.

[3] Anagaw, Y. K., Ayenew, W., Limenh, L. W., Geremew, D. T., Worku, M. C., Tessema, T. A., Simegn, W., Mitku, M. L. (2024): Food adulteration: causes, risks, and detection techniques. – SAGE Open Medicine 12: 20503121241250184.

[4] Artavia, G., Cortés-Herrera, C., Granados-Chinchilla, F. (2021): Selected instrumental techniques applied in food and feed: quality, safety and adulteration analysis. – Foods 10: 1081.

[5] Barathan, M., Ng, S. L., Lokanathan, Y., Ng, M. H., Law, J. X. (2024): The profound influence of gut microbiome and extracellular vesicles on animal health and disease. – International Journal of Molecular Sciences 25: 4024.

[6] Basak, S., Shetty, P. H. (2021): Conventional Microbial Counting and Identification Techniques. – In: Khan, M. S., Shafiur Rahman, M. (eds.) Techniques to Measure Food Safety and Quality. Springer, Cham, pp. 69-89.

[7] Bryden, W. L. (2012): Mycotoxin contamination of the feed supply chain: implications for animal productivity and feed security. – Animal Feed Science and Technology 173: 134-158.

[8] Bukin, Y. S., Mikhailov, I. S., Petrova, D. P., Galachyants, Y. P., Zakharova, Y. R., Likhoshway, Y. V. (2023): The effect of metabarcoding 18S rRNA region choice on diversity of microeukaryotes including phytoplankton. – World Journal of Microbiology and Biotechnology 39: 229.

[9] Bylemans, J., Gleeson, D. M., Hardy, C. M., Furlan, E. (2018): Toward an ecoregion scale evaluation of eDNA metabarcoding primers: a case study for the freshwater fish biodiversity of the Murray–Darling Basin (Australia). – Ecology and Evolution 8: 8697-8712.

[10] Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Peña, A. G., Goodrich, J. K., Gordon, J. I. (2010): QIIME allows analysis of high-throughput community sequencing data. – Nature Methods 7: 335-336.

[11] Cerf, M. E. (2021): Healthy Lifestyles and Noncommunicable Diseases: Nutrition, the Life-Course, and Health Promotion. – Lifestyle Medicine. https://doi.org/10.1002/lim2.31.

[12] Cheli, F., Battaglia, D., Pinotti, L., Baldi, A. (2012): State of the art in feedstuff analysis: a technique-oriented perspective. – Journal of Agricultural and Food Chemistry 60: 9529-9542.

[13] Choi, J., Park, J. S. (2020): Comparative analyses of the V4 and V9 regions of 18S rDNA for the extant eukaryotic community using the Illumina platform. – Scientific Reports 10: 6519.

[14] Čolović, R., Puvača, N., Cheli, F., Avantaggiato, G., Greco, D., Đuragić, O., Kos, J., Pinotti, L. (2019): Decontamination of mycotoxin-contaminated feedstuffs and compound feed. – Toxins 11: 617.

[15] d'Enfert, C., Kaune, A.-K., Alaban, L.-R., Chakraborty, S., Cole, N., Delavy, M., Kosmala, D., Marsaux, B., Frois-Martins, R., Morelli, M. (2021): The impact of the Fungus-Host-Microbiota interplay upon Candida albicans infections: current knowledge and new perspectives. – FEMS Microbiology Reviews 45: fuaa060.

[16] Dagnas, S., Membré, J.-M. (2013): Predicting and preventing mold spoilage of food products. – Journal of Food Protection 76: 538-551.

[17] Ducrot, C., Barrio, M. B., Boissy, A., Charrier, F., Even, S., Mormède, P., Petit, S., Schelcher, F., Casabianca, F., Ducos, A. (2024): Animal board invited review: improving animal health and welfare in the transition of livestock farming systems: towards social acceptability and sustainability. – Animal 101100.

[18] Edgar, R. C. (2013): UPARSE: highly accurate OTU sequences from microbial amplicon reads. – Nature Methods 10: 996-998.

[19] FAO (2023): Sustainable Livestock Transformation—A Vision for FAO’s Work on Animal Production and Health. – FAO, Rome.

[20] Feye, K., Baxter, M., Tellez-Isaias, G., Kogut, M., Ricke, S. (2020): Influential factors on the composition of the conventionally raised broiler gastrointestinal microbiomes. – Poultry Science 99: 653-659.

[21] Field, K. G., Olsen, G. J., Lane, D. J., Giovannoni, S. J., Ghiselin, M. T., Raff, E. C., Pace, N. R., Raff, R. A. (1988): Molecular phylogeny of the animal kingdom. – Science 239: 748-753.

[22] Flynn, J. M., Brown, E. A., Chain, F. J., MacIsaac, H. J., Cristescu, M. E. (2015): Toward accurate molecular identification of species in complex environmental samples: testing the performance of sequence filtering and clustering methods. – Ecology and Evolution 5: 2252-2266.

[23] Fonseca, V., Kirse, A., Giebner, H., Vause, B., Drago, T., Power, D., Peck, L., Clark, M. (2022): Metabarcoding the Antarctic Peninsula biodiversity using a multi-gene approach. – ISME Communications 2: 37.

[24] Fu, C., Ni, J., Huang, R., Gao, Y., Li, S., Li, Y., Zhong, K., Zhang, P. (2023): Sex different effect of antibiotic and probiotic treatment on intestinal microbiota composition in chemically induced liver injury rats. – Genomics 115: 110647.

[25] Gao, J., Yin, J., Xu, K., Li, T., Yin, Y. (2019): What is the impact of diet on nutritional diarrhea associated with gut microbiota in weaning piglets: a system review. – BioMed Research International 2019: 6916189.

[26] Garcia-Bustos, V., Rosario Medina, I., Cabañero Navalón, M. D., Ruiz Gaitán, A. C., Pemán, J., Acosta-Hernández, B. (2024): Candida spp. in Cetaceans: neglected emerging challenges in marine ecosystems. – Microorganisms 12: 1128.

[27] Gurikar, C., Shivaprasad, D., Sabillón, L., Gowda, N. N., Siliveru, K. (2023): Impact of mycotoxins and their metabolites associated with food grains. – Grain Science and Technology 6: 1-9.

[28] Hassoun, A., Guðjónsdóttir, M., Prieto, M. A., Garcia-Oliveira, P., Simal-Gandara, J., Marini, F., Di Donato, F., D’Archivio, A. A., Biancolillo, A. (2020): Application of novel techniques for monitoring quality changes in meat and fish products during traditional processing processes: reconciling novelty and tradition. – Processes 8: 988.

[29] Hou, K., Wu, Z.-X., Chen, X.-Y., Wang, J.-Q., Zhang, D., Xiao, C., Zhu, D., Koya, J. B., Wei, L., Li, J. (2022): Microbiota in health and diseases. – Signal Transduction and Targeted Therapy 7: 1-28.

[30] Hugerth, L. W., Muller, E. E., Hu, Y. O., Lebrun, L. A., Roume, H., Lundin, D., Wilmes, P., Andersson, A. F. (2014): Systematic design of 18S rRNA gene primers for determining eukaryotic diversity in microbial consortia. – PLoS ONE 9: e95567.

[31] Ibarra, R., Rich, K. M., Adasme, M., Kamp, A., Singer, R., Atlagich, M., Estrada, C., Jacob, R., Zimin-Veselkoff, N., Escobar-Dodero, J. (2018): Animal production, animal health and food safety: gaps and challenges in the Chilean industry. – Food Microbiology 75: 114-118.

[32] Imanian, B., Donaghy, J., Jackson, T., Gummalla, S., Ganesan, B., Baker, R. C., Henderson, M., Butler, E. K., Hong, Y., Ring, B. (2022): The power, potential, benefits, and challenges of implementing high-throughput sequencing in food safety systems. – NPJ Science of Food 6: 35.

[33] Janik, E., Niemcewicz, M., Podogrocki, M., Ceremuga, M., Gorniak, L., Stela, M., Bijak, M. (2021): The existing methods and novel approaches in mycotoxins’ detection. – Molecules 26: 3981.

[34] Kassa, M. G., Habtu, N. G., Worku, A. F., Abera, B. D., Asemu, A. M. (2023): Effect of Threshing and Storage Conditions on Mold Contamination of Stored Maize Grain (Zea mays L.). – In: Mequanint, K. et al. (eds.) Advancement of Science and Technology in Sustainable Manufacturing and Process Engineering. Springer, Cham, pp. 261-281.

[35] Khan, A. H., Karuppayil, S. M. (2012): Fungal pollution of indoor environments and its management. – Saudi Journal of Biological Sciences 19: 405.

[36] Kim, S. W., Less, J. F., Wang, L., Yan, T., Kiron, V., Kaushik, S. J., Lei, X. G. (2019): Meeting global feed protein demand: challenge, opportunity, and strategy. – Annual Review of Animal Biosciences 7: 221-243.

[37] Kolde, R. (2018): pheatmap: Pretty Heatmaps. – R package version 1.0.12.

[38] Larsson, J., Godfrey, A. J. R., Gustafsson, P., Eberly, D. H., Huber, E., Privé, F. (2024): eulerr: Area-Proportional Euler and Venn Diagrams with Ellipses. – R Package Version 7.0.2.

[39] Lejzerowicz, F., Esling, P., Pillet, L., Wilding, T. A., Black, K. D., Pawlowski, J. (2015): High-throughput sequencing and morphology perform equally well for benthic monitoring of marine ecosystems. – Scientific Reports 5: 13932.

[40] Li, Q., Fei, H.-L., Luo, Z.-H., Gao, S.-M., Wang, P.-D., Lan, L.-Y., Zhao, X.-F., Huang, L.-N., Fan, P.-F. (2023): Gut microbiome responds compositionally and functionally to the seasonal diet variations in wild gibbons. – NPJ Biofilms and Microbiomes 9: 21.

[41] Liu, Z., Liu, M., Meng, J., Wang, L., Chen, M. (2024): A review of the interaction between diet composition and gut microbiota and its impact on associated disease. – Journal of Future Foods 4: 221-232.

[42] Magoč, T., Salzberg, S. L. (2011): FLASH: fast length adjustment of short reads to improve genome assemblies. – Bioinformatics 27: 2957-2963.

[43] Mamphogoro, T., Mpanza, T., Mani, S. (2024): Animal Feed Production and Its Contribution to Sustainability of Livestock Systems: African Perspective. – Nciizah, A. D. et al. (eds.) The Marginal Soils of Africa: Research, Utilization, Management, and Reclamation. Springer, Cham, pp. 37-54.

[44] Medeiros, F., Aleman, R. S., Gabríny, L., You, S. W., Hoskin, R. T., Moncada, M. (2024): Current status and economic prospects of alternative protein sources for the food industry. – Applied Sciences 14: 3733.

[45] Meulenbelt, S. (2018): Assessing chemical, biological, radiological and nuclear threats to the food supply chain. – Global Security: Health, Science, and Policy 3: 14-27.

[46] Mohapatra, D., Kumar, S., Kotwaliwale, N., Singh, K. K. (2017): Critical factors responsible for fungi growth in stored food grains and non-chemical approaches for their control. – Industrial Crops and Products 108: 162-182.

[47] Momtaz, M., Bubli, S. Y., Khan, M. S. (2023): Mechanisms and health aspects of food adulteration: a comprehensive review. – Foods 12: 199.

[48] Mottet, A., de Haan, C., Falcucci, A., Tempio, G., Opio, C., Gerber, P. (2017): Livestock: on our plates or eating at our table? A new analysis of the feed/food debate. – Global Food Security 14: 1-8.

[49] Nelson, C. E. (1993): Strategies of mold control in dairy feeds. – Journal of Dairy Science 76: 898-902.

[50] Ni, J., Yan, Q., Yu, Y., Zhang, T. (2014): Factors influencing the grass carp gut microbiome and its effect on metabolism. – FEMS Microbiology Ecology 87: 704-714.

[51] Ni, J., Fu, C., Huang, R., Li, Z., Li, S., Cao, P., Zhong, K., Ge, M., Gao, Y. (2021): Metabolic syndrome cannot mask the changes of faecal microbiota compositions caused by primary hepatocellular carcinoma. – Letters in Applied Microbiology 73: 73-80.

[52] Pakdel, M., Olsen, A., Bar, E. M. S. (2023): A review of food contaminants and their pathways within food processing facilities using open food processing equipment. – Journal of Food Protection 86: 100184.

[53] Parisi, G., Tulli, F., Fortina, R., Marino, R., Bani, P., Dalle Zotte, A., De Angelis, A., Piccolo, G., Pinotti, L., Schiavone, A. (2020): Protein hunger of the feed sector: the alternatives offered by the plant world. – Italian Journal of Animal Science 19: 1204-1225.

[54] Paulk, R. T., Abbas, H. K., Rojas, M. G., Morales-Ramos, J., Busman, M., Little, N., Shier, W. T. (2024): Evaluating Acheta domesticus (Orthoptera: Gryllidae) for the

reduction of fumonisin B1 levels in livestock feed. – Journal of Economic Entomology 117: 427-434.

[55] Pexas, G., Doherty, B., Kyriazakis, I. (2023): The future of protein sources in livestock feeds: implications for sustainability and food safety. – Frontiers in Sustainable Food Systems 7: 1188467.

[56] Poutanen, K. S., Karlund, A. O., Gómez-Gallego, C., Johansson, D. P., Scheers, N. M., Marklinder, I. M., Eriksen, A. K., Silventoinen, P. C., Nordlund, E., Sozer, N. (2022): Grains—a major source of sustainable protein for health. – Nutrition Reviews 80: 1648-1663.

[57] Predescu, N. C., Stefan, G., Rosu, M. P., Papuc, C. (2024): Fermented feed in broiler diets reduces the antinutritional factors, improves productive performances and modulates gut microbiome—a review. – Agriculture 14: 1752.

[58] R Core Team (2021): R: A Language and Environment for Statistical Computing. – R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

[59] Ren, Z., Zhang, X., Fan, H., Yu, Y., Yao, S., Wang, Y., Dong, Y., Deng, H., Zuo, Z., Deng, Y. (2023): Effects of different dietary protein levels on intestinal aquaporins in weaned piglets. – Journal of Animal Physiology and Animal Nutrition 107: 541-555.

[60] Rico-Munoz, E., Samson, R. A., Houbraken, J. (2019): Mould spoilage of foods and beverages: using the right methodology. – Food Microbiology 81: 51-62.

[61] Santos Pereira, C., Cunha, S., Fernandes, J. O. (2019): Prevalent mycotoxins in animal feed: occurrence and analytical methods. – Toxins 11: 290.

[62] Sforza, S., Dall'Asta, C., Marchelli, R. (2006): Recent advances in mycotoxin determination in food and feed by hyphenated chromatographic techniques/mass spectrometry. – Mass Spectrometry Reviews 25: 54-76.

[63] Shu, L., Ludwig, A., Peng, Z. (2021): Environmental DNA metabarcoding primers for freshwater fish detection and quantification: in silico and in tanks. – Ecology and Evolution 11: 8281-8294.

[64] Stewart, S. C., Dritz, S. S., Woodworth, J. C., Paulk, C., Jones, C. K. (2020): A review of strategies to impact swine feed biosecurity. – Animal Health Research Reviews 21: 61-68.

[65] Stoeck, T., Bass, D., Nebel, M., Christen, R., Jones, M. D., Breiner, H. W., Richards, T. A. (2010): Multiple marker parallel tag environmental DNA sequencing reveals a highly complex eukaryotic community in marine anoxic water. – Molecular Ecology 19: 21-31.

[66] Sui, L., Li, J., Philp, J., Yang, K., Wei, Y., Li, H., Li, J., Li, L., Ryder, M., Toh, R. (2022): Trichoderma atroviride seed dressing influenced the fungal community and pathogenic fungi in the wheat rhizosphere. – Scientific Reports 12: 9677.

[67] Thornton, P., Gurney-Smith, H., Wollenberg, E. (2023): Alternative sources of protein for food and feed. – Current Opinion in Environmental Sustainability 62: 101277.

[68] Tuomilehto, J. (2022): How to prevent non-communicable diseases? A continuous need for a better understanding of the role of nutritional factors through scientific research. – European Journal of Clinical Nutrition 76: 1357-1363.

[69] van der Poel, A. F. B., Abdollahi, M. R., Cheng, H., Čolović, R., den Hartog, L. A., Miladinovic, D., Page, G., Sijssens, K., Smillie, J. F., Thomas, M., Wang, W., Yu, P., Hendriks, W. H. (2020): Future directions of animal feed technology research to meet the challenges of a changing world. – Animal Feed Science and Technology 270: 114692.

[70] Vaulot, D., Geisen, S., Mahé, F., Bass, D. (2022): pr2-primers: an 18S rRNA primer database for protists. – Molecular Ecology Resources 22: 168-179.

[71] Wickham, H. (2016): ggplot2: Elegant Graphics for Data Analysis. – Springer, New York.

[72] Wu, G., Bazer, F. W., Cross, H. R. (2014): Land-based production of animal protein: impacts, efficiency, and sustainability. – Annals of the New York Academy of Sciences 1328: 18-28.

[73] Wu, S., Zhang, X., Dai, L., Ni, J., Wang, Y., Li, J., Ding, W. (2023): Differences of copepoda composition analysis in mariculture ponds through morphological taxonomy and different metagenetic approaches. – Applied Ecology and Environmental Research 21(3): 2091-2131.

[74] Wu, Y., Wang, B., Tang, L., Zhou, Y., Wang, Q., Gong, L., Ni, J., Li, W. (2022): Probiotic Bacillus alleviates oxidative stress-induced liver injury by modulating gut-liver axis in a rat model. – Antioxidants 11: 291.

[75] Xiao, X., Zou, P.-R., Hu, F., Zhu, W., Wei, Z.-J. (2023): Updates on plant-based protein products as an alternative to animal protein: technology, properties, and their health benefits. – Molecules 28: 4016.

[76] Xiong, F., Shu, L., Zeng, H., Gan, X., He, S., Peng, Z. (2022): Methodology for fish biodiversity monitoring with environmental DNA metabarcoding: the primers, databases and bioinformatic pipelines. – Water Biology and Security 1: 100007.

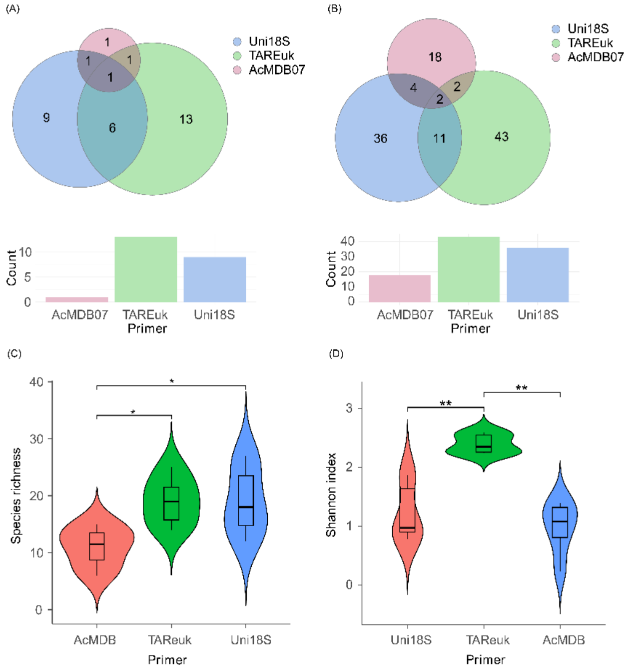
[77] Xu, H., Zhang, S., Ma, G., Zhang, Y., Li, Y., Pei, H. (2020): 18S rRNA gene sequencing reveals significant influence of anthropogenic effects on microeukaryote diversity and composition along a river-to-estuary gradient ecosystem. – Science of the Total Environment 705: 135910.

[78] Zhan, A., Hulák, M., Sylvester, F., Huang, X., Adebayo, A. A., Abbott, C. L., Adamowicz, S. J., Heath, D. D., Cristescu, M. E., MacIsaac, H. J. (2013): High sensitivity of 454 pyrosequencing for detection of rare species in aquatic communities. – Methods in Ecology and Evolution 4: 558-565.

[79] Zheng, X., He, Z., Wang, C., Yan, Q., Shu, L. (2022): Evaluation of different primers of the 18S rRNA gene to profile amoeba communities in environmental samples. – Water Biology and Security 1: 100057.

APPENDIX

Appendix 1. *Venn and violin diagrams show the difference of species identifications obtained by three different primer pairs. (A) Venn diagram at phylum level; (B) Venn diagram at species level; (C) Difference of species richness; (D) Difference of Shannon index. * P < 0.05; ** P < 0.01*



Appendix 2. *Species compositions in the feed and feed raw materials detected using three different primer pairs*

Species	S1Uni18S	S2Uni18S	S3Uni18S	S4Uni18S	S5Uni18S	S6Uni18S
Candida saopauloensis	0.4039	0.7922	0.8425	0.7714	0.5405	0.7946
Saccharomyces cerevisiae	0.2517	0.0002	0.0002	0.0004	0.0014	0.0000
Other	0.0361	0.0321	0.0381	0.0362	0.0639	0.0347
Aspergillus penicillioides	0.0055	0.0100	0.0136	0.0525	0.1080	0.0287
Pseudoplatyophrya nana	0.0131	0.0837	0.0111	0.0278	0.0321	0.0448
Geotrichum candidum	0.0247	0.0140	0.0339	0.0328	0.0433	0.0413
Komagataella phaffii	0.1197	0.0000	0.0000	0.0004	0.0000	0.0000
Colpoda steinii	0.0148	0.0239	0.0193	0.0148	0.0238	0.0126
Unidentified	0.0252	0.0005	0.0000	0.0356	0.0381	0.0047
[Candida] intermedia	0.0072	0.0192	0.0057	0.0000	0.0035	0.0161
Anas platyrhynchos	0.0000	0.0000	0.0062	0.0128	0.0287	0.0000
[Candida] saopauloensis	0.0034	0.0077	0.0104	0.0072	0.0091	0.0088
Triticum monococcum	0.0426	0.0000	0.0000	0.0000	0.0000	0.0000
Glycine max	0.0261	0.0000	0.0000	0.0000	0.0000	0.0000
Pelmatolapia mariae	0.0000	0.0000	0.0000	0.0000	0.0220	0.0000
Sinistrostrombidium cupiformum	0.0000	0.0000	0.0000	0.0000	0.0122	0.0079
Scopulariopsis crassa	0.0000	0.0000	0.0000	0.0002	0.0171	0.0016
Brassica napus	0.0002	0.0150	0.0000	0.0000	0.0003	0.0000
Uncultured Gregarina	0.0000	0.0000	0.0000	0.0000	0.0147	0.0000
Aspergillus insolitus	0.0002	0.0000	0.0000	0.0004	0.0133	0.0003
Candida parapsilosis	0.0075	0.0000	0.0032	0.0000	0.0014	0.0000
Diutina sipiczkii	0.0009	0.0000	0.0079	0.0000	0.0003	0.0000
Pichia kudriavzevii	0.0013	0.0000	0.0005	0.0000	0.0052	0.0000
Paraleptospora chromolaenicola	0.0009	0.0000	0.0005	0.0017	0.0017	0.0019
Pseudodiaptomus annandalei	0.0055	0.0000	0.0000	0.0000	0.0000	0.0000
Diutina catenulata	0.0000	0.0000	0.0000	0.0000	0.0052	0.0000
Uncultured ciliate	0.0000	0.0000	0.0000	0.0052	0.0000	0.0000
Glycine soja	0.0047	0.0000	0.0000	0.0000	0.0000	0.0000
Nannochloropsis oceanica	0.0005	0.0015	0.0007	0.0004	0.0014	0.0000
Hyphopichia burtonii	0.0000	0.0000	0.0000	0.0000	0.0042	0.0000
Gallus gallus	0.0000	0.0000	0.0000	0.0000	0.0042	0.0000
Alphamonas edax	0.0004	0.0000	0.0015	0.0002	0.0000	0.0019
Lichtheimia corymbifera	0.0000	0.0000	0.0007	0.0000	0.0031	0.0000
Oreochromis niloticus	0.0000	0.0000	0.0035	0.0000	0.0000	0.0000
Cladosporium sp.	0.0013	0.0000	0.0005	0.0000	0.0010	0.0000
Sinocalanus sinensis	0.0027	0.0000	0.0000	0.0000	0.0000	0.0000

Species	S1TAReuk	S2TAReuk	S3TAReuk	S4TAReuk	S5TAReuk	S6TAReuk
Pseudodiaptomus annandalei	0.0338	0.2267	0.1062	0.2305	0.1646	0.1807
Desmodesmus arthrodesmiformis	0.0301	0.2400	0.1947	0.0974	0.1519	0.1506
Other	0.0752	0.1067	0.1416	0.1136	0.0759	0.0843
Anchoa hepsetus	0.0000	0.0000	0.0708	0.0325	0.2532	0.1386

Saccharomyces cerevisiae	0.3609	0.0000	0.0000	0.0000	0.0000	0.0000
Asplanchna Brightwellii	0.0000	0.0400	0.0619	0.1071	0.0506	0.0904
Unidentified	0.0150	0.0667	0.0619	0.0974	0.0380	0.0602
Binuclearia lauterbornii	0.0150	0.0933	0.0796	0.0519	0.0253	0.0723
Paracyclopina nana	0.0075	0.0267	0.0088	0.0325	0.0633	0.0723
Penaeus vannamei	0.1805	0.0000	0.0000	0.0000	0.0000	0.0000
Nephrocytium agardhianum	0.0038	0.0400	0.0088	0.0487	0.0127	0.0361
Uncultured ciliate	0.0000	0.0000	0.0442	0.0260	0.0000	0.0241
Glycine soja	0.0414	0.0400	0.0000	0.0000	0.0000	0.0000
Sinocalanus sinensis	0.0113	0.0000	0.0000	0.0097	0.0506	0.0000
Trachelomonas grandi	0.0714	0.0000	0.0000	0.0000	0.0000	0.0000
Uncultured Hafniomonas	0.0000	0.0133	0.0000	0.0292	0.0000	0.0241
Pompholyx sulcata	0.0150	0.0000	0.0000	0.0227	0.0127	0.0120
Pelagostrobilidium minutum	0.0000	0.0000	0.0531	0.0000	0.0000	0.0060
Uncultured eukaryote	0.0000	0.0000	0.0265	0.0260	0.0000	0.0060
Orientocreadium pseudobagri	0.0451	0.0000	0.0088	0.0000	0.0000	0.0000
Gallus gallus	0.0000	0.0000	0.0000	0.0032	0.0506	0.0000
Pelmatolapia mariae	0.0000	0.0000	0.0000	0.0000	0.0380	0.0060
Strombidium guangdongense	0.0000	0.0000	0.0354	0.0065	0.0000	0.0000
Malva sylvestris	0.0000	0.0400	0.0000	0.0000	0.0000	0.0000
Tanichthys albonubes	0.0000	0.0000	0.0088	0.0097	0.0000	0.0181
Simulium tani	0.0338	0.0000	0.0000	0.0000	0.0000	0.0000
Artemia sp. NT99	0.0000	0.0000	0.0265	0.0065	0.0000	0.0000
Ficopomatus shenzhensis	0.0000	0.0267	0.0000	0.0032	0.0000	0.0000
Peronospora belbahrii	0.0000	0.0000	0.0265	0.0000	0.0000	0.0000
Desmodesmus komarekii	0.0000	0.0133	0.0000	0.0130	0.0000	0.0000
Dolerocypria taalensis	0.0038	0.0000	0.0000	0.0000	0.0127	0.0060
Metschnikowia bicuspidata	0.0000	0.0133	0.0088	0.0000	0.0000	0.0000
Brassica napus	0.0075	0.0133	0.0000	0.0000	0.0000	0.0000
Monoraphidium sp. HDMA-11	0.0000	0.0000	0.0177	0.0000	0.0000	0.0000
Follicularia paradoxalis	0.0075	0.0000	0.0000	0.0097	0.0000	0.0000
Cephalodella forficula	0.0000	0.0000	0.0088	0.0065	0.0000	0.0000
Itunella muelleri	0.0000	0.0000	0.0000	0.0032	0.0000	0.0120
Nannochloropsis oceanica	0.0150	0.0000	0.0000	0.0000	0.0000	0.0000
Trachelomonas sp.	0.0113	0.0000	0.0000	0.0000	0.0000	0.0000
Triticum aestivum	0.0075	0.0000	0.0000	0.0000	0.0000	0.0000
Chytridiales sp.	0.0075	0.0000	0.0000	0.0000	0.0000	0.0000
Rhizophlyctis rosea	0.0000	0.0000	0.0000	0.0065	0.0000	0.0000
Picochlorum sp. RCC748	0.0000	0.0000	0.0000	0.0065	0.0000	0.0000

Species	S1AcMDB	S2AcMDB	S3AcMDB	S4AcMDB	S5AcMDB	S6AcMDB
Engraulis ringens	0.6451	0.0045	0.3576	0.7100	0.4550	0.7478
Rhinogobio cylindricus	0.1343	0.9548	0.0134	0.0125	0.0025	0.0065
Oreochromis niloticus	0.0046	0.0000	0.4154	0.0757	0.1703	0.2172
Anas platyrhynchos	0.0077	0.0000	0.1147	0.1020	0.2073	0.0008

Gallus gallus	0.0000	0.0000	0.0600	0.0783	0.1462	0.0016
Tachysurus fulvidraco	0.1265	0.0249	0.0103	0.0110	0.0010	0.0081
Homo sapiens	0.0710	0.0015	0.0000	0.0000	0.0000	0.0008
Other	0.0077	0.0030	0.0066	0.0045	0.0047	0.0081
Channa maculata	0.0000	0.0000	0.0114	0.0016	0.0050	0.0065
Ictalurus punctatus	0.0031	0.0000	0.0051	0.0016	0.0035	0.0008
Spinibarbus hollandi	0.0000	0.0113	0.0000	0.0000	0.0000	0.0000
Anser cygnoides	0.0000	0.0000	0.0053	0.0015	0.0038	0.0000
Pangasianodon hypophthalmus	0.0000	0.0000	0.0000	0.0005	0.0001	0.0008
Acanthaluteres Brownii	0.0000	0.0000	0.0000	0.0002	0.0002	0.0008
Cairina moschata	0.0000	0.0000	0.0002	0.0002	0.0003	0.0000
Scomber australasicus	0.0000	0.0000	0.0000	0.0000	0.0003	0.0000
Sardina pilchardus	0.0000	0.0000	0.0000	0.0002	0.0000	0.0000
Anas poecilorhyncha	0.0000	0.0000	0.0000	0.0002	0.0000	0.0000