Aquaculture Reports 36 (2024) 102031

Contents lists available at ScienceDirect

Aquaculture Reports

journal homepage: www.elsevier.com/locate/aqrep

New metagenomic procedure for the investigation of the eukaryotes present in the digestive gland of *Mytillus galloprovincialis*

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A R T I C L E I N F O

Key words:
Mytilus galloprovincialis
Mussel diet
Eukaryotic microbiome
Metabarcoding
NGS

A B S T R A C T

Mussels' aquaculture is one of the most important cultures in Europe, with a high level of self-sufficiency. In Spain, the main cultivated species is *M. galloprovincialis*, whose production takes place primarily in Galicia. In that region, a Protected Designation of Origin certification exists to certify the high quality of the product and its sustainability. This species is also present in other regions making the control of its traceability complicated. In order to distinguish Galician mussels from the others, the eukaryotes present in its digestive gland could be a useful tool. However, to use them as biomarkers, having an adapted protocol to recover the diversity present in the digestive gland is essential. In this work, we have selected a new protocol to optimize the study of the diversity of eukaryotes associated with the digestive gland of *M. galloprovincialis*. This protocol is based on a combination of a pre-treatment and a commercial DNA extraction kit, the amplification of DNA with two newly designed primer sets to avoid the DNA from the mussel, and the use of next-generation sequencing. The results showed a significant reduction of mussel DNA amplification and enough DNA quality for the subsequent sequencing, increasing the number of sequences recovered from other eukaryotes. This new protocol offers then a good chance to determine the diversity of eukaryotes present in the digestive gland of *M. galloprovincialis*, and it can be used in future studies to examinate the seasonal variation of the present species and to evaluate its potential as geographical traceability tool.

1. Introduction

In 2018, shelled mollusk aquaculture represented 56.3 % (17.3 million tonnes) of the world production of marine and coastal aquaculture (FAO, 2020). European Union (EU) is the largest single market for bivalves (FAO, 2018), in which the sustained high prices have driven the expansion of bivalves' aquaculture industry in various regions (FAO, 2020). Mussel aquaculture is one of the most important and the only species for which EU holds a high level of self-sufficiency (EUMOFA, 2019), defined as the ratio between own production (catches plus aquaculture) and total apparent consumption (European Commission). Almost 94 % of the world mussel production comes from aquaculture and its production represents more than a third of the EU aquaculture production (Avdelas et al., 2021). In 2017, the EU mussel production reached 464,240 tonnes with a total value of EUR 423 million (EUMOFA, 2019). Within Europe, Spain is the major producer of mussel, followed by France and Italy (FAO, 2020), these three countries representing more than half of the total production of mussels in terms of volume. The Mediterranean mussel (*Mytilus galloprovincialis*) is the main cultivated species in Spain, and 97 % of its production takes place in Galicia (North-West region of Spain), where its extensive aquaculture is based on ropes suspended from floating rafts (Irisarri et al., 2015; del rio-Lavín et al., 2022a). The mussel products from this area have a Protected Designation of Origin (PDO) since 2007, certifying its excellent quality and traceability according to the EU seafood policy standards (Azpeitia et al., 2017).

To meet the EU traceability standards, besides a correct identification of species, the authentication of the product origin is essential, even more in the case of a product with PDO certification as *M. galloprovincialis* from Galicia. This mussel species is also cultivated in

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https://doi.org/10.1016/j.aqrep.2024.102031
Received 6 June 2023; Received in revised form 3 March 2024; Accepted 12 March 2024
Available online 18 March 2024

other regions of the Spain, as Catalonia, Valencia, Balearic Islands, or Andalusia (Ramón et al., 2005; https://www.mapa.gob.es/es/pesca/temas/acuicultura/produccion-de-acuicultura/produccion-ccaa/), as well as in other regions of the world as Ireland, Portugal and French Atlantic coast (Wenne et al., 2022), the northern shores of the Mediterranean Sea (France, Italy, Albania, Greece, Croatia, Bosnia, Slovenia, Turkey, Bulgaria; FAO, 2009), some southern Mediterranean countries (Morocco, Argelia, Tunisia), the Russian Federation, Ukraine (FAO, 2009), Japan and USA (Braby and Somero, 2006), Namibia or South Africa (Zardi et al., 2018), New Zeland and Australia (Wenne et al., 2022). This species is also farmed in China (FAO, 2009) and Chile, although in the latter only experimental and regulated cultivation is allowed, being *M. galloprovincialis* considered an invasive species (Díaz et al., 2019). Given the wide distribution of the aquaculture of this species, the only way to validate the origin declared on the labels is to have tools able to establish the geographical origin and to differentiate the mussels produced in different regions.

Different techniques have been used to establish the geographical origin of mussels. Molecular methods based on genetic markers have been used to discriminate the different species within *Mytilus* genus (Santaclara et al., 2006), but the variability found was not enough to discriminate the different Mediterranean mussel populations. Other methods based on the differences imposed by different environmental conditions, as chemical characterization of organic, mineral or isotope composition or analysis of minor and trace elements have been also widely used for discriminating bivalves from different regions (e.g., Costas-Rodriguez et al., 2010; Ricardo et al., 2015; del Rio-Lavín et al., 2022b). The study of the diet and/or the microbiome (eukaryotes and prokaryotes) associated with the species, or its populations have also been used to establish geographical patterns (e.g., Parlapania et al., 2018; Liu et al., 2020; Feng et al., 2021). In this line, next-generation sequencing (NGS) has opened new possibilities, allowing to know the organisms associated with the species or included in its diet in just one analysis (Yoon et al., 2017). This kind of studies have demonstrated to be very informative regarding the geographic origin in some aquatic species, such as plaice (Morris et al., 2021), Manila clam (Milan et al., 2019) or soft-shell clam (Liu et al., 2020). Other microbiome study based on NGS was also applied successfully to discriminate between farmed and wild mussels (Santibañez et al., 2022). However, these techniques have not yet been tested to the study of the species present in the digestive gland of *M. galloprovincialis*, in order to establish its geographical origin. These species are expected to be a mix of prokaryotes and eukaryotes that mussels have filtered as part of their diet together with those that constitute their microbiome. Some information has been reported about the bacterial community associated with the digestive gland of the mussels (e.g. Vezzulli et al., 2017). However, there is hardly any information about the eukaryotes present in their digestive gland. The eukaryotes study entails one extra challenge since the mussel is also an eukaryote. If universal primers for eukaryotes are used, the DNA from the mussel will be amplified together with the eukaryotes present in the diet and microbiome, overthrowing a large part of the sequencing. This can result in a too low number of NGS reads to resolve the eukaryote species associated with the mussel digestive gland. For this reason, in this work we have developed a new procedure to study the eukaryote organisms present in the digestive gland of *M. galloprovincialis*, based on: (1) a new DNA extraction protocol with a pre-treatment that reinforce the recovery of eukaryote organisms; (2) an amplification procedure for eukaryotes DNA avoiding the amplification of the host mussel DNA by using new developed universal primers. This complete procedure would be able to successfully recover and amplify enough DNA for the NGS study of the eukaryotic community associated with the digestive gland of the mussels without the meddling of mussel DNA.

2. Material and methods

2.1. Sample pre-treatment and DNA extraction from digestive gland

Twenty-seven adult *M. galloprovincialis* individuals were collected in June 2019 from a mussel farm in Ria de Arousa (Galicia, north-west of Spain), one of the areas with the greatest production of this species in Europe, mainly using floating rafts (20 × 25 m) (Fig. 1). The individuals were preserved alive in porexpan box with ice packs until arrival at the laboratory. After removing the shells, the bodies of the mussels, were randomly divided into three groups of nine individuals each, to be subjected to 3 different pre-treatments. Mussels from the first group were fixed in ethanol (EF) performing the following steps: 2 mL of 95 % ethanol were injected directly into the digestive gland, then it was kept at 4 °C overnight in a 50 mL tube at the fridge. Then, the mussel was submerged in 70 % ethanol for two hours. Finally, the mussel was put in a petri dish and the digestive gland was separated using a scalpel, under a stereomicroscope (EZ4 HD, Leica Microsystems). The separated gland was then used for the DNA extraction. The second group was subjected to three rounds of freeze-thaw cycles (FT): first, the digestive gland was separated with a scalpel under a stereomicroscope and immediately frozen at −80 °C by putting the tube in the freezer for one hour, and then it was heated in a constant temperature bath at 95 °C for 15 min. This freeze-thaw round was repeated a second time. Finally, it was frozen at −80 °C in the freezer overnight and heated for 15 min the next morning. After this treatment, the DNA extraction was carried out. The last group of samples was processed combining both pre-treatments (EF-FT).

After pre-treatment, three DNA extraction protocols were tested: (1) DNeasy Blood and Tissue Kit (Qiagen) using the Gram-positive bacteria protocol, (2) DNeasy PowerSoil Kit (Qiagen), following the manufacturers' instructions, and (3) DNeasy PowerSoil Kit modified in the first step with an extra lysis buffer (30 M Tris, 10 mM EDTA, 1 % SDS), specifically 60 μL of this new buffer and 60 μL of C1 buffer in the kit were added. The rest of the process was performed as indicated by the manufacturer. DNA from nine samples was extracted with each protocol, three from each pre-treatment, following the scheme showed in the Fig. 2. In addition, one negative control (water instead of tissue) per protocol was included in order to monitor any possible contamination during the extraction procedure. The extracted DNA was quantified by fluorimetry using Qubit 3.0 (Life Technologies) with Qubit™ dsDNA HS/BR Assay Kits (ThermoFisher Scientific).

2.2. Primers design

Two new primer sets were designed to amplify as many as possible eukaryotic species that might constitute the mussel diet and therefore could be present in the digestive gland. These primers were intended to

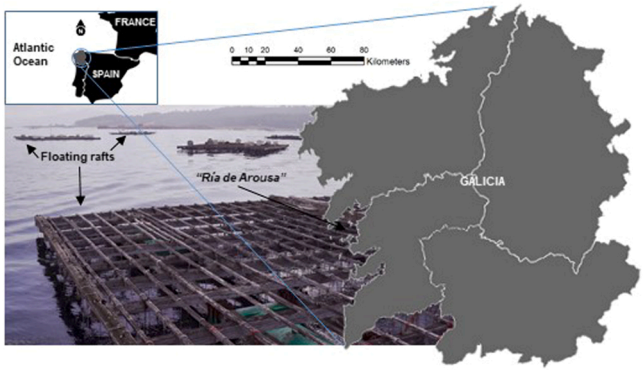

Fig. 1. Map showing Ría de Arousa location in the Galicia coastline (North-west of Spain). Detail of floating rafts from a mussel farm where *Mytillus galloprovincialis* individuals are cultivated.

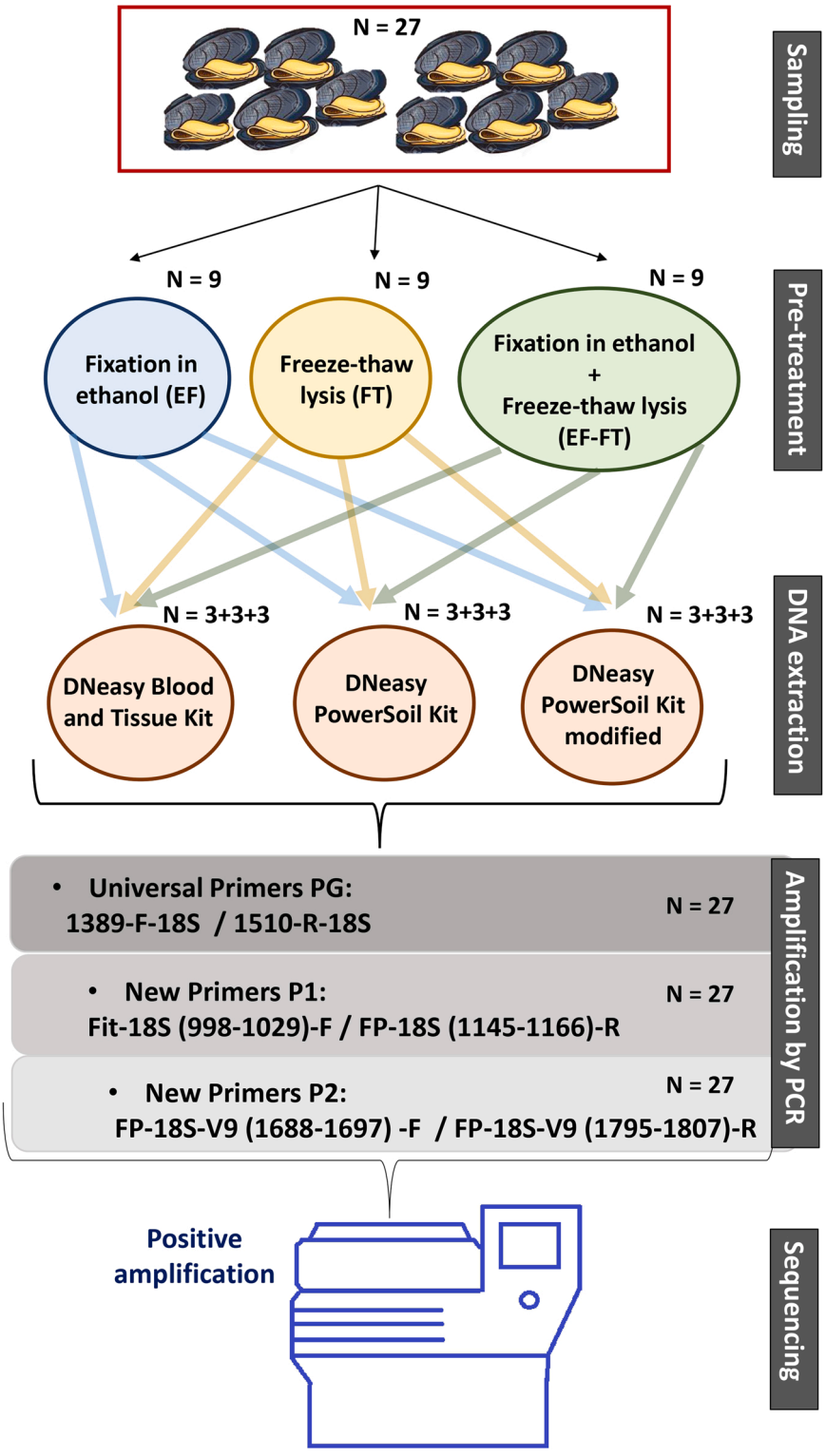

Fig. 2. Summary of the workflow performed in the laboratory to sequence the DNA present in the digestive gland of mussels.

amplify just eukaryotic plankton species while avoiding the amplification of the mussel DNA, in order to improve the performance of the subsequent sequencing. For this purpose, nine 18S rDNA sequences from common plankton species included in 8 families and 6 different orders (supplementary Table S1) were downloaded from GenBank for making a multiple sequence alignment with CLUSTALW (Thompson et al., 1994). Additionally, one 18 S rDNA sequence of mussel *Mytilus* (L24490.1) was also downloaded.

The newly designed primers were

Fit-18S (998–1029) F-5′ATCAAGAACGAAAGTDDGGGGW-3′, with melting temperature (Tm) between 51.1 – 54.8 °C, and FP-18S (1145–1166) R TTTCAGCCTTGTGACCATACTC-3′, with Tm 53 °C, hereinafter called primer set P1, which amplify a fragment of V5 region of 18 S rDNA (about 160 bp length).

FP-18S-V9 (1688–1697) F-5′-CCTACCGATTGARTGGTCCG-3′, with Tm 53.8 – 55.9 °C, and FP-18S-V9 (1795–1807) R-5′-

GAAACCTTGTTACGAYTTCWC-3′, with Tm 48.5 – 50.5 °C, hereinafter called primer set P2, which amplify a fragment of V9 region of 18 S rDNA (about 120 bp length). Both primer sets and specific locations in the reference alignment can be seen in the supplementary Fig. S1. These primers were tested for checking the absence of amplification when using DNA extracted from mussel foot tissue as control.

2.3. Amplification and sequencing

For each extracted sample (N = 27) three different PCRs were carried out: one with each newly designed couple of primers (P1 and P2), and other with the eukaryotic universal primers developed by Amar-al-Zettler et al. (2009), which amplify a fragment of 18 S rDNA gene in the V9 hypervariable region:

1389-F-18S 5'-TTGTACACACCGCCC-3′ and 1510-R-18S 5'-CCTTCYGCAGGTTCACCTAC-3′). This fragment (87–186 bp) was used as a control of the species found in the digestive gland since the amplification of mussel DNA is not hindered, and these primers hereinafter are called general primers (PG). Each PCR was carried out in 25 μL volume using the DreamTaq Hot Start PCR Master Mix (ThermoFisher Scientific) following the manufacturer's conditions and 25–50 ng of DNA. The best annealing conditions (Tm) of the newly designed primers was investigated by a gradient PCR. The final PCR program was 94 °C for 15 min, 35 cycles of 94 °C for 30 sec, 53 °C for 30 s, and 72 °C for 1 min, with a final extension step of 72 °C for 7 min, in a Veriti Thermal Cycler (Applied Biosystems), for P1 and P2. We used 35 cycles to ensure the amplification of possible species that were present but more degraded or in low proportion in the sample, since the DNA present in the digestive gland is likely degraded due to the digestion process. In the case of universal primers, the PCR protocol was the same except for the annealing temperature, which was 57 °C. Reamplification of the initial PCR products was needed to get enough DNA for next-generation sequencing. We made an internal comparison, contrasting the results of the positive sample, amplified with universal primers, with the results obtained for the same sample, with the same primers but using 30 cycles, like in the original publication is done. We did not find big differences in the community recovered (data not shown) therefore we assumed that the possible increase of bias due to the use of 35 cycles instead 30 cycles can be neglected.

Besides DNA extracted from digestive gland samples, the negative controls of the DNA extraction (one per DNA extraction protocol tested defined above), DNA extracted from a plankton sample from 1 L filtered water using a 2 μm pore-size filter (Millipore) as positive control, and a negative PCR control (water replaced the DNA template), were also included in the PCR.

All PCR products were run in a 2 % agarose gel stained with RedSafe nucleic acid staining solution (20,000X, Intron Biotechnology) to assess the amplification. The PCR products with positive amplification were purified using "AMPure XP" reagent (Beckman Coulter) and quantified using Qubit 3.0 with dsDNA HS Assay Kit. The amplicon libraries were prepared with the "Ion Plus Fragment Lybrary kit" (ThermoFisher) and the "Ion express barcode adapters 1–16" (ThermoFisher), following the manufacturers' instructions. The libraries were purified using "AMPure XP" reagent and were doubly quantified: by fluorimetry, using Qubit 3.0 and dsDNA HS Assay Kit, and by qPCR, using the Ion Library TaqMan Quantification Kit (ThermoFisher), in a 7900HT Fast Real-Time PCR System (Applied Biosystems) following the manufacturer instructions. In addition, a quality control of size was performed with an Agilent 2100 Bioanalyzer. Barcoded libraries were combined into two pools with a final concentration of 8 pM, then they were loaded in two Ion 316™ Chip v2 BC (Life Technologies) using the reagents included in the Ion PGM Hi-Q Chef Kit (Life Technologies). The sequencing was performed on Ion PGM™ sequencer (Life Technologies) with 500 flows. The initial processing of data from PGM runs was performed automatically by the Ion PGM software, removing adaptors, barcodes, and poor signal reads and obtaining one fastq file per sample and amplicon.

2.4. Analysis of sequences

First quality checking of the sequences was performed with FastQC program (Andrews, 2010). Then the sequences were filtered by quality and minimum length, using the thresholds Q20 and 80 bp as minimal length, with Cutadapt software (Martin, 2011). The denoising, dereplication and feature-table construction were performed in QIIME2 v2020.8 (Bolyen et al., 2019) using the plugin "qiime dada2 denoise--pyro". Afterwards the taxonomic assignment was carried out with the plugin "qiime feature-classifier classify-consensus-blast" (Camacho et al., 2009), with 97 % of identity, 90 % coverage, and both strands checked as settings, and using the Silva 132 database (Quast et al., 2013). All features with less than 10 sequences were removed. The plankton sample reads, amplified with the three primer sets and used as a positive control, were normalized by rarefaction and analyzed separately from the digestive gland samples. A barplot was constructed with QIIME2 for plankton samples to check the main differences in taxa amplification according to the primers used. The percentage of unassigned sequences was calculated for both, plankton and digestive gland samples, and these sequences were removed. For the digestive gland samples, the percentage of sequences that belonged to Mytiloida order was calculated. The means of unassigned and Mytiloida sequences by primer were compared using Mann-Whitney test or T-student test, when the data met normality, using R software (R Core Team, 2020). Richness (observed taxa) and Simpson and Shannon entropy indexes were calculated with QIIME2 and drawn with R studio v3.6.2 (2019–12–12). These values were compared among the samples amplified with different primers and among the samples extracted with different protocols using Mann Whitney test (R studio). Beta diversity based on Bray Curtis matrix was also calculated using QIIME2 and Principal Coordinate Analysis (PCoA) and it was plotted with R. Anosim test was used to check if the community amplified with the three primer sets was significantly different and Kruskal-Wallis test and LEfSe analysis (Linear discriminant analysis Effect Size) (Segata et al., 2011) were used to find out which taxa were recovered with significantly different abundances according to the new primer sets, P1 or P2.

3. Results

3.1. Pre-treatments, DNA extraction and amplification

The quantity of DNA extracted was very variable among protocols (understood as the combination of pre-treatment and DNA extraction method) and variable among samples (Table1; supplementary Table S2) with high standard deviations. The samples in which the combination of pre-treatments was used (EF-FT), resulted in low DNA concentration and the lowest yield. The highest values were obtained when the combination of ethanol fixation with Power Soil kit was used (EF-PS). However, when ethanol fixation (EF) pre-treatment was applied, the highest standard deviations were obtained, with one out of three samples quite different for each DNA extraction method. In fact, this pre-treatment combined with the modification of Power Soil (EF-PM) was negative with lower DNA concentration recovered and a standard deviation even higher than the mean. Using this pre-treatment, only EF-PS combination amplified with the two newly designed sets of primers. The other two protocols that resulted in positive amplification were those which combined Freeze-Thaw lysis and Blood & Tissue (FT-BT) or Power Soil kit (FT-PS), respectively. On the contrary, all samples could be amplified with the PG set of primers, which amplify eukaryotes DNA including mussel.

Summarizing, only nine combinations (pre-treatment & DNA extraction method) showed positive amplification, 9 with PG, 9 with P2, and 8 with P1 (sample 20 (FT-BT) did not amplify with this primer set), making a total of 26 samples, plus the plankton control, that were sequenced in the next step. Negative controls did not show any amplification to continue with the sequencing. Table 1

Table 1
Mean and standard deviation of DNA concentration (ng/μL) and DNA yield (ng/mg) for each protocol tested. EF = Fixation in ethanol; FT = Freeze-Thaw lysis; BT = Blood & Tissue kit; PS = Power Soil kit; PM = Power soil kit modified. The protocols from which was possible to obtain positive amplification are in bold.

	EF			FT			EFFT		
	BT	PS	PM	BT	PS	PM	BT	PS	PM
DNA extract (ng/μL)	14.3 ± 7.35	**79.5 ± 38.95**	7.1 ± 10.72	**5.8 ± 2.98**	**3.7 ± 1.86**	1.0 ± 0.82	4.9 ± 2.99	0.2 ± 0.04	0.3 ± 0.16
DNA yield (ng/mg)	2.4 ± 1.21	**34.1 ± 16.83**	1.8 ± 2.75	**1.0 ± 0.49**	**1.3 ± 0.67**	0.3 ± 0.22	0.8 ± 0.35	0.1 ± 0.01	0.3 ± 0.12

3.2. Sequencing results and potential of the new primers

The quality of the sequences obtained was good (> Q20) in all samples amplified with the three sets of primers. After all filters (quality, minimal length and denoising) and the taxonomical assignment, the positive control samples (marine plankton control) were normalized to 95,520 sequences, and the digestive gland samples were normalized to 99,144 sequences for subsequent analysis.

3.2.1. Positive control sample results

The positive control sample (marine plankton) was amplified with the three pairs of primers and the results were compared to check if the newly designed primers produced biased amplification resulting in the loss of some group of eukaryotic plankton species in addition of avoiding the amplification of the mussel DNA. The percentage of unassigned sequences was 16.76 % for PG, 6.09 % for P1 and 35.26 % for P2 sets. Most groups were amplified similarly by the new primers compared with the general primers, except Metazoa group, which was not amplified by P2 primers and subtly amplified by P1 primers (Fig. 3). Other groups not amplified in this sample by P2 primers were Cercozoa, Picozoa or Choanoflagelida, but these taxa, except Cercozoa, were found in low abundance with PG and P1 primers. On the other hand, P1 primers did not present sequences classified at less specific taxonomic levels such as "SAR" (Stramenopiles, Alveolata, Rhizaria) or "Eukaryota". An additional advantage of the newly designed primers appears to be that they do not amplify bacteria or archaea (Fig. 3), only a very low proportion of bacteria from the Planctomycetes group were amplified by P1 primers (represented as bacteria taxonomic group). This seems to help to increase the proportion of eukaryotic organisms classified in some groups, such as Chlorophyta, Cryptomonadales, Dinoflagellata (for both primers set) or Cercozoa (for P1 set).

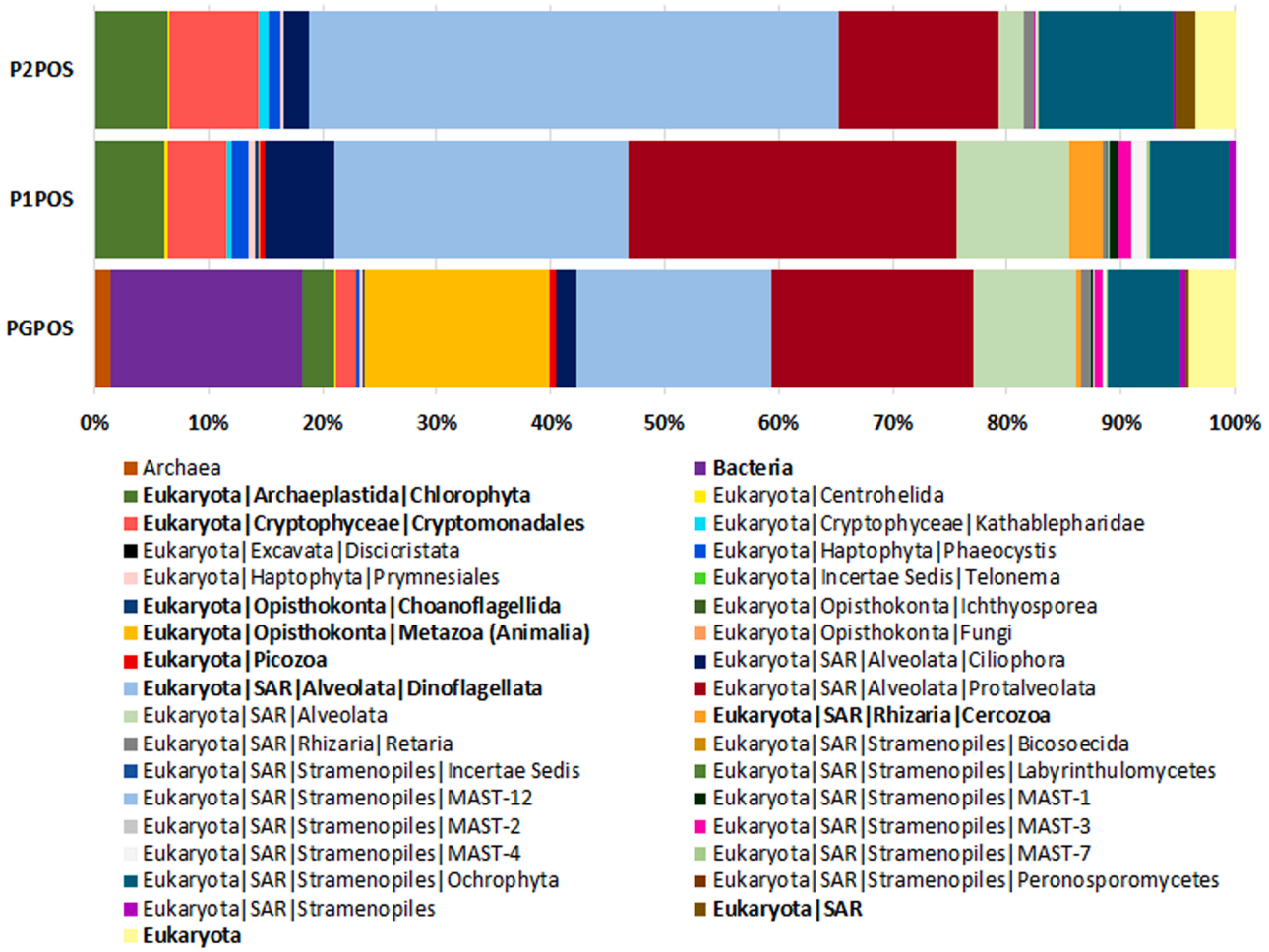

Fig. 3. Bar plot showing the relative abundances of the taxonomical groups found in the positive control sample amplified with each of the primers. (PGPOS) Positive control sample amplified with the general primers, (P1POS) positive control sample amplified with the new P1 primers, and (P2POS) positive control sample amplified with the new P2 primers. Unassigned sequences were removed before drawing the bar plot. The taxonomical groups mentioned in the text are marked in bold.

3.2.2. Digestive gland samples' results

The mean percentage of unassigned sequences was 0.418 % for PG, mainly due to the extremely high proportion of Mytiloida sequences (Fig. 4). For P1 and P2 sets, the mean percentages of unassigned sequences were 49.064 % and 38.607 % respectively and the difference was not significant. Mean percentages of sequences assigned to Mytiloida order were 83.745 %, 0.005 % and 0.004 % for the samples amplified with PG, P1 and P2 primers respectively (Fig. 4). The difference between P1 and P2 was not significant but both resulted in a significant reduction of the Mytiloida DNA amplification compared to PG [PG-P1 ($W = 72$; p-value <0.001) and PG-P2 ($W = 81$; p-value <0.001)].

When the alpha diversity was analysed, the samples amplified with the newly designed primers (P1 and P2) showed higher diversity than samples amplified with PG primers in all cases (Fig. 5), and this difference was significant [Observed features: PG vs. P1 ($W = 10$, p-value <0.02); Shannon entropy: PG vs. P1 ($W = 0$, p-value <0.001), PG vs. P2 ($W = 3$, p-value <0.001); Simpson index: PG vs. P1 ($W = 0$, p-value <0.001), PG vs. P2 ($W = 3$, p-value <0.001)], except for the comparison of Observed features between PG versus P2. Alpha diversity values were very similar among protocols for Shannon and Simpson indexes in the case of samples amplified with PG and P2 primers (supplementary Fig. S2). However, for samples amplified with P1 primers, higher alpha diversity values were obtained for the samples extracted with the protocol FT-BT, but these differences were not significant.

The community recovered with the three couples of primers was different according to the PCoA, where the differences between the communities recovered with PG and those recovered with P1 or P2 explained 44 % of the variability, and the differences between the communities recovered with P1 respect to P2 explained 16 % of the variability (supplementary Fig. S3). These differences were corroborated by the significant anosim test ($R = 0.935$, p-value $= 0.001$). The sample P220 was represented separated to the rest (supplementary Fig. S3), due to the overrepresentation of Gymnodinium clade (Dinoflagellata) in that sample (Fig. 6), so it was considered as an outlier.

The comparison of the community recovered with the P1 and P2 primers was performed after removing the samples amplified with PG, mainly formed by Mytiloida sequences. Some taxa were exclusively or significantly more amplified depending on the primers used during amplification according to the Kruskal-Wallis test (p-value < 0.05) (Fig. 7A). Specifically, Alveolata, Cercozoa, Chlorophyta and Stramenopiles taxa showed higher abundances when P1 primers were used, in addition to Pirellulaceae bacteria (Planctomycetes bacteria). On the other hand, some dinoflagellata like Gymnodiniphycidae had higher abundance when P2 primers were used (5 % more recovered with P2). When the same comparison was performed with LEfSe test, significant differences (p-value < 0.05) were found only in favor of P1 primers, showing similar results to Kruskal-Wallis test but adding Opisthokonta as other group more amplified with P1 primers and not including Stramenopiles taxa (Fig. 7B and C).

Regarding the DNA extraction protocol applied in each sample, no pattern was found for the samples amplified with P1 primers, but the two samples extracted with FT-BT protocol (19 and 21) appeared slightly separated (Fig. 6A). These two samples were the only ones where Cryptomycota, and Saccharomycetaceae fungi, Peritrichia and Ochromonadales were amplified with P1 primers and they showed significantly more abundance of Syndiniales (supplementary Table S3). On the other hand, samples extracted with FT-PS seemed to be more homogeneous (Fig. 6A) and showed the highest content of Dinoflagellata (supplementary Table S3). The samples amplified with P2 primers showed a weak pattern, separating the samples extracted with FT-BT protocol from the samples extracted with FT-PS protocol and explaining 15 % of variability (Fig. 6A). Samples extracted with FT-BT protocol were the only ones in which Charophyta, Oligotrichia and Fucales were amplified using P2 primers (supplementary Table S3). In addition, those samples presented the highest content of Syndiniales and the samples extracted with FT-PS the highest abundance for Dinoflagellata when the P2 primes were used. The samples extracted with EF-PS protocol appeared distributed into both groups and showed the highest abundance for Ochrophyta. This group was only amplified in these samples and in those extracted with FT-BT protocol.

Finally, comparing the samples extracted with the same DNA extraction protocol but different primers, there were no significant differences in the relative abundances of taxonomic groups found in samples extracted with FT-BT protocol and different primers. However, the samples extracted with EF-PS and FT-PS did show differences between primers (supplementary Table S4). Chlorodendrales, Alveolata and Thraustochytriaceae were more abundant in the samples amplified with P1 primers for both DNA extraction protocols whereas some Dinoflagellata were more abundant in samples amplified with P2. In addition, for EF-PS protocol, other Dinoflagellata, Syndiniales and Ochrophyta were more represented in the samples amplified with P2 primers and Pirellulaceae bacteria, Thecofilosea and Mamiellophyceae were slightly more represented when P1 primers were used. Finally, for FT-PS some Stramenopiles, Ebriacea and Mytiloida showed higher abundance when P1 primers were used.

4. Discussion

In this work a new protocol to optimize the study of eukaryote organisms associated with the digestive gland of *M. galloprovincialis* using

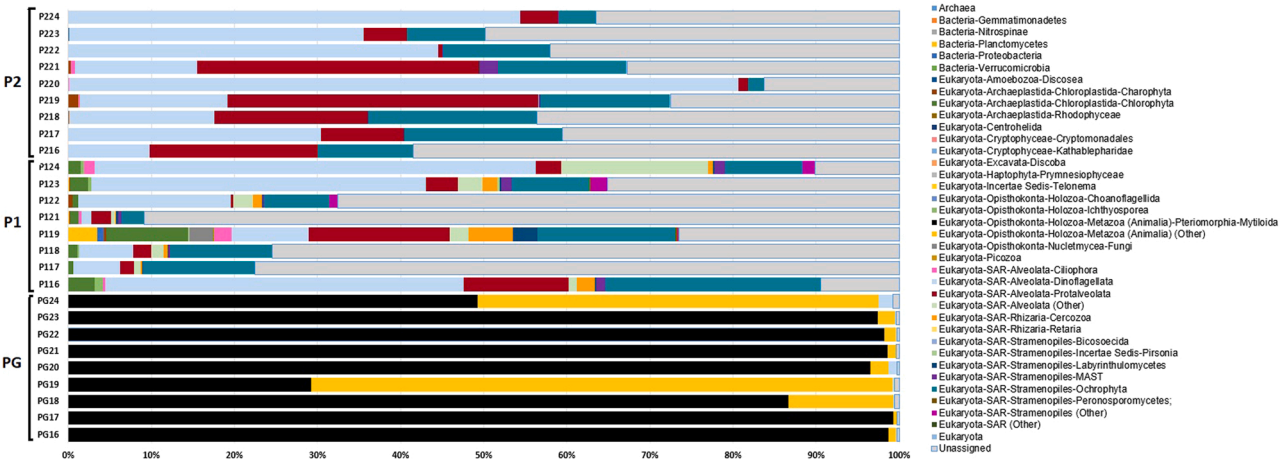

Fig. 4. Bar plot of the relative abundances of taxa found in the digestive gland samples amplified with the three couple of primers. (PG) samples amplified with the general primers, (P1) samples amplified with the new P1 primers and (P2) samples amplified with the new P2 primers.

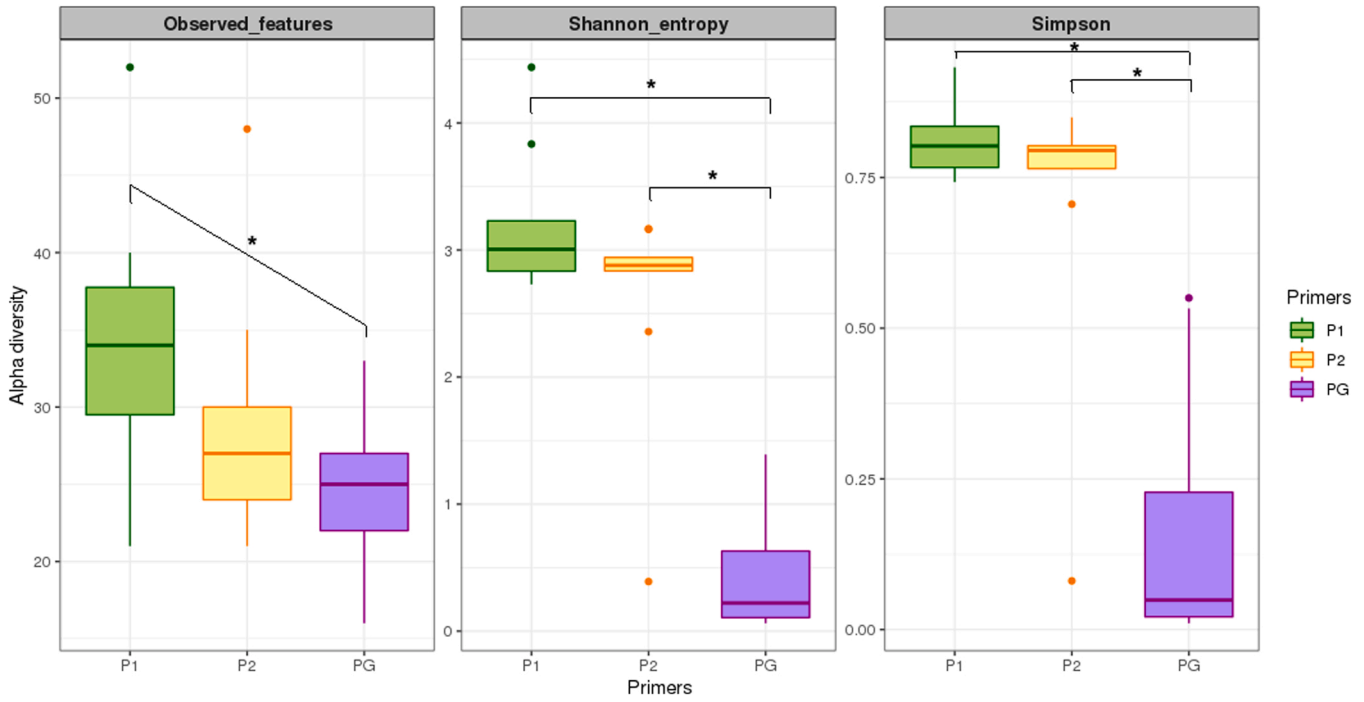

Fig. 5. Alpha diversity of the samples represented by primers set. Richness (Observed features), Shannon entropy and Simpson index are shown. The comparisons that resulted in significant differences are marked with an asterisk.

NGS methodology has been developed, with the objective to offer new alternatives to study its diet and its eukaryote microbiome. This new protocol is based on a commercial DNA extraction kit combined with a pre-treatment to reinforce the eukaryote's DNA recovery, and newly designed phytoplankton universal primer sets able to avoid mussel DNA amplification, without losing diversity of the community of eukaryotic organisms associated with the digestive gland.

Three different pre-treatments of the digestive gland were studied with each DNA extraction method to favor the release of DNA from the eukaryotic organisms ingested by the mussel and its eukaryotic microbiome. Mussels feed by filtering very different types of organisms present in the water, basically organisms belonging to the micro and nanoplankton categories (Frojan et al., 2014). Some of these organisms, such as diatoms, present cell walls made of silica that may be difficult to break. Moreover, the digestive gland of mussels is a dense glandular tissue, and this may also difficult the recovery of DNA from the organisms present there. The use of a pre-treatment before the DNA extraction helps to detach and break the eukaryotic cells from the glandular tissues as well as to preserve the DNA quality. The fixation of the tissues with ethanol in different steps of the pretreatment has been successfully used by other authors (e.g.: Maloy et al., 2009) to improve the DNA extraction yield of the marine plankton community associated with the digestive gland of different mollusks, therefore conserving the diversity present in it. Fixation with ethanol has been described as a useful method to use with animal tissues before DNA extraction, yielding high quality and non-fragmented DNA (Panzacchi et al., 2019). Results found here also shows that this pre-treatment of digestive gland seems to produce higher yields compared with the other two, but the variability was the highest independently of DNA extraction kit used. Additionally, with this pre-treatment only the samples extracted with Power Soil kit could be amplified, indicating that despite of the yield was higher, the DNA was mainly from the mussel and not from the other eukaryotes present in the digestive gland since all samples were successfully amplified with PG primers but no with P1 and P2 primers. On the contrary, the second pre-treatment applied, cycles of extreme temperature changes (FT), produced a lower DNA yield than the EF pre-treatment although more samples could be amplified with the newly designed primers (FT-BT and FT-PS samples). Other researchers already obtained good results using freeze-thaw cycles to recover the microbiome present in digestive gland of the oyster *Saccostrea glomerata* (Green and Barnes, 2010). Bigot-Clivot et al. (2020) also obtained good quality DNA of protozoa applying six cycles of −80 °C for 5 min and 95 °C for 4 min over mussel's haemolymph.

On the other hand, two out of three protocols with positive amplification were a combination of a pre-treatment with Power Soil kit (PS). This kit has been widely selected for NGS analysis of water samples and water sediments since the results obtained with this method were more comparable and consistent than with other extraction methods, being successfully used for the extraction of DNA from environmental samples in different matrices (Walden et al., 2017; Pearman et al., 2020). This commercial kit can efficiently remove potential PCR inhibitors or contaminants from challenging samples thanks to its patented Inhibition Removal Technology and it is able to bind enough DNA to its silica columns to continue the downstream analysis such as NGS, where the minimum amount of DNA required for a successful process is higher than for other techniques (Pearman et al., 2020). So, the first objective of obtaining enough good quality DNA from digestive gland to study the eukaryotic organisms present through NGS was reached by means FT as pre-treatment and/or PS as DNA extraction kit. When we explored the distribution of the samples according to the community of eukaryotes recovered, the samples processed with the FT-PS protocol were more homogeneous than those processed with FT-BT, independently of the primer set used, and the homogeneity of the samples processed with one protocol is very important.

Regarding the amplification of the DNA from the organisms present in a complex sample, understood as a sample composed of a mixture of different organisms, besides the extracted DNA having a good quality and the right average template size, the primers chosen should also have the annealing sites in the most eukaryotic target. When universal primers are used to amplify a sample with diverse species, the predominant and less degraded DNA will be amplified preferentially (Leray et al., 2013). This is a common problem when analysing components of diet or microbiome studies in eukaryote organisms, where the DNA of the host species is often present in great excess in the samples (Vestheim

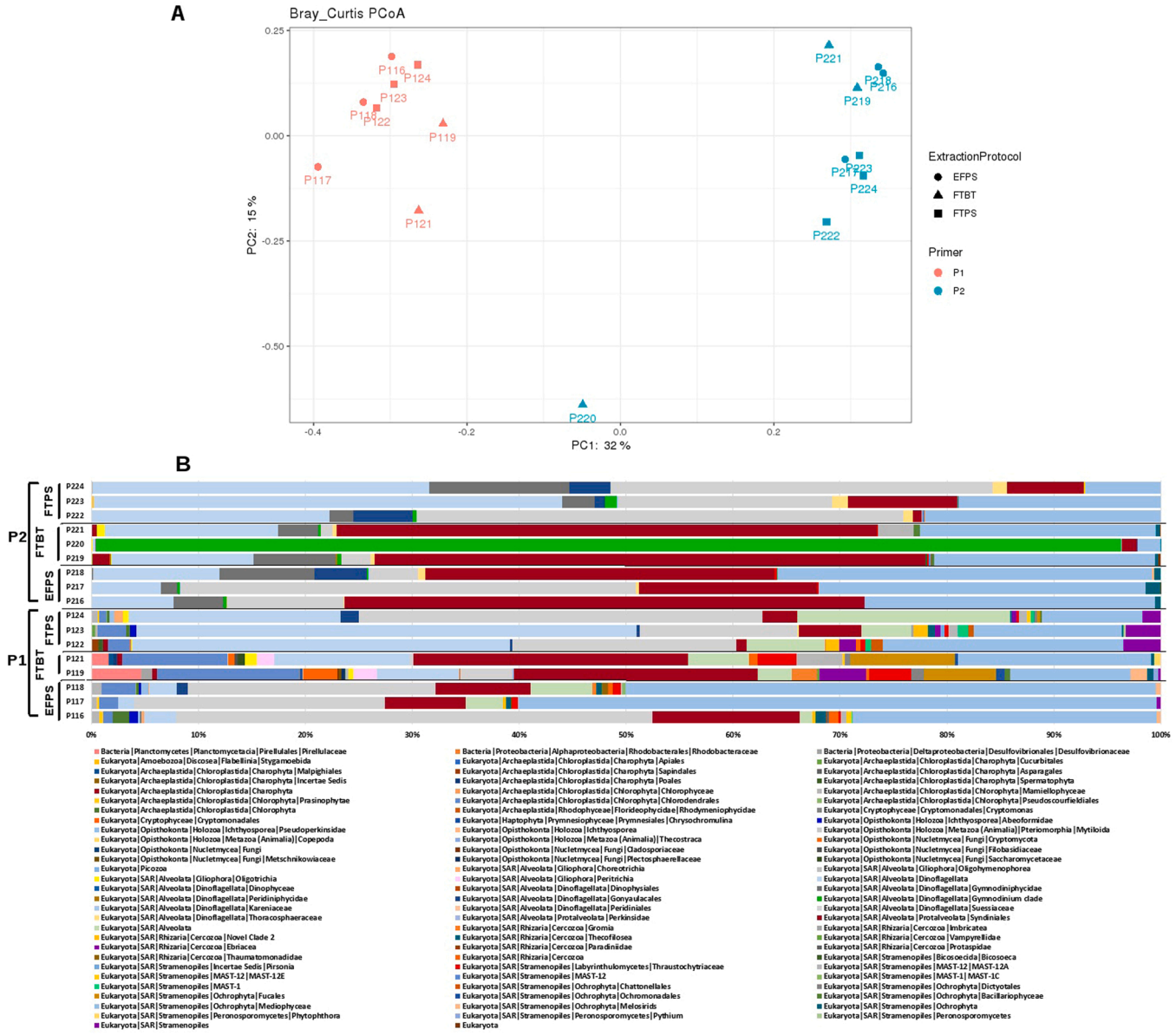

Fig. 6. Eukaryotic community recovered from digestive gland of *M. galloprovincialis*. (A) PCoA representing the samples amplified with P1 and P2 primers. The color shows the primers used and the shape represents the DNA extraction protocol applied in each sample. (B) Bar plot representing the relative abundances of the taxa found in each sample when they were amplified with P1 and P2 primers. The samples are sorted by DNA extraction protocol within each couple of primers used.

and Jarman, 2008). Because of that, the use of blocking primers or modified or newly designed specific primers, is necessary in order to exclude the confounding host template DNA.

Here, using the newly designed primer sets (P1 and P2), the percentage of Mytiloida reads was significantly reduced (10,000 folds) and non-amplification of bacteria was also confirmed for P2 and P1, except a negligible amplification of Pirellulales group with P1. Therefore, the second objective of avoiding non-target organisms to favour the recovery of other target species present was reached with both new primer sets. The sequencing optimization based on improving the specificity of the primers has been proven as very efficient in other works, modifying the primers (Hadziavdic et al.,2014; Minardi et al., 2022), as in this case, or using blocking primers to avoid non-target amplifications from the host eukaryote (Vestheim and Jarman, 2008; Leray et al., 2013; Arenz et al., 2015). Designing new primers has also the advantage that additional primers shouldn't be added to the PCR, as in the case of blocking primers, which could interfere with the specific PCR system. Either way, avoiding non-target DNA amplification, like bacteria or host mussel DNA in this case, the amplification becomes more efficient (Minardi et al., 2022), providing NGS capacity for the detection of lower represented target plankton species reads that can be part of mussel diet, thus better representing the diversity present in the sample. This is reflected in our results, with a significant increase of the alpha diversity when P1 or P2 are used, compared with the PG results. Additionally, the amplification of some taxonomical groups was promoted, like Chlorophyta or some Dinoflagellata.

Concerning to the potential bias of the new primer sets, no mayor bias was detected using P1 and P2, regarding the taxonomy and diversity of the community amplified, compared to the PG primers for the control plankton sample. Limiting the universality of the primers might introduce biases and exclude important groups from the analysis (Lanzen et al., 2011) and it should be checked. Additionally, the potential bias related with the degeneration of the primers should also be evaluated. For checking all these bias we amplified with P1 and P2 and sequenced the positive sample. That sample was a plankton sample from filtered water, so we expected high diversity and similar community

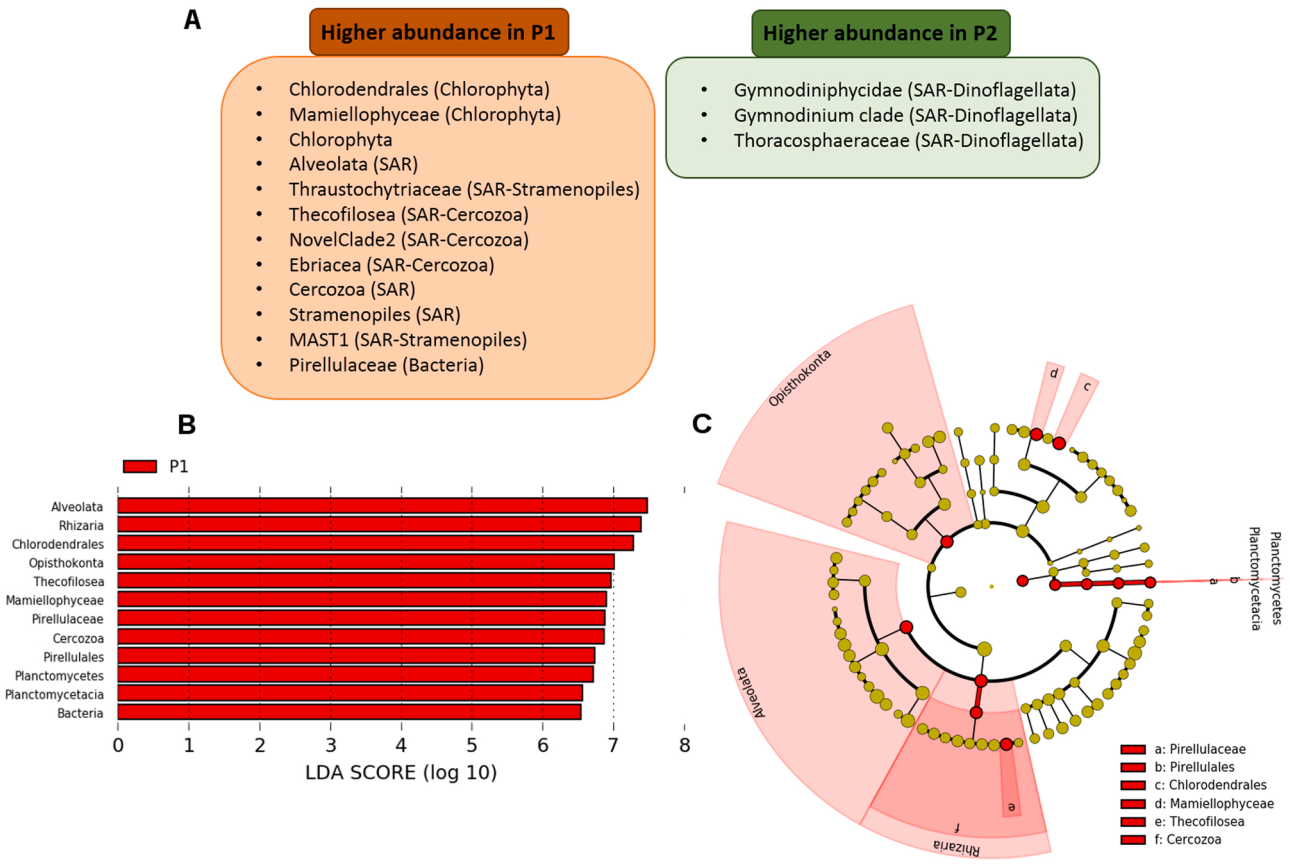

Fig. 7. Differences between newly designed primers P1 and P2. (A) Taxonomic groups with significant differences in their relative abundances (p-value<0.05) depending on the primers used in the amplification step. (B) Results of LEfSe analysis showing the taxonomic groups with significantly different relative abundances (p-value<0.05). The established LDA score threshold was 2.0. (C) Cladogram associated to the LEfSe results.

than that recovered with the PG universal primer set developed by Amaral-Zettler et al. (with less ambiguous nucleotides) if no important bias was present. Confirming what we expected, only the Metazoa group, which includes Mytiloida, was slightly amplified with P1, while it was not with P2. Metazoa group is formed mainly by larvae phases of animals that will be out of plankton fraction after their larvae phases, like fishes, echinoderms, etc. Mussels' diet is mainly phytoplankton and organic material (Babarro et al., 2019), so the loss of Metazoa group in the amplification is not a critical limitation to study the diet and symbionts associated to the mussels' digestive gland. In fact, other authors have already glimpsed the potential advantages of designing "anti--metazoan" primers for the study of associated eukaryotes (diet, symbionts, parasites, etc.) with other larger eukaryotes (Carnegie et al., 2003; Bower et al., 2004; Hugerth et al., 2014; Bass and del Campo, 2020; Minardi et al., 2022). In this case, it is clear that using P1 and P2 can offer a better representation of the eukaryote diversity associated with the digestive gland of the mussel *M. galloprovincialis*.

Regarding the diversity obtained with the new sets of primers, no significant differences were found between them. Only weak differences were presented in the taxonomical composition for the plankton control sample, with Cercozoa, Picozoa and Choanoflagelida not detected by P2. However, those groups appeared in very low abundances also using P1 and PG for the control sample and the last two were also not amplified in any of the digestive gland samples. Cercozoa, although present in low abundance, was only amplified by P1 set, as well as other groups like Alveolata, Chlorophyta, Stramenopiles or Opisthokonta which also obtained higher abundance when P1 set was used. In contrast, the Dinoflagellate group (different organisms) was more abundantly detected when the P2 set was used. But all these groups, except Cercozoa, could be amplified with both primer sets.

Mussels, as filter feeders, consume plankton and other organic and inorganic suspended particles in the surrounding environment (Newell, 2004; Frojan et al., 2014), but in coastal areas they rely mostly on phytoplankton (Wai and Levinton, 2004). However, some previous works showed that, mussels could present a selective feeding behaviour and some significant differences were found between the species present in the surrounding water and the ones present in the digestive system of the mussel. For instance, Rouillon et al. (2005) showed that the dominant species present in the digestive system of the blue mussel were diatoms and dinoflagellates and our results reproduce that finding for *M. galloprovincialis* from Galicia. For example, Mediophyceae (diatoms) and Suessiaceae or Syndiniales (dinoflagellate), as well as other dinoflagellate with more taxonomical levels not resolved were found in high proportion in all gland samples. However, the proportion of species found in the digestive gland can vary according to different geographical regions, depending on the organisms present in water, making the microbiome a possible, useful, tool to traceability. Milan et al. (2019) were able to discriminate geographical origin in *Ruditapes philippinarum*, studying the bacterial microbiome associated with digestive gland. Singh et al. (2023) used bacterial microbiome associated with the gills of *Crassostrea virginica* to achieve the same goal. Elaised et al. (2019) could discriminate different niches of *Oreochromis niloticus* using their gut content. The protocol selected here allows to effectively recover the biodiversity present in the digestive gland of the mussels, opening the possibility to perform for *M. galloprovincialis* studies similar to previously mentioned, to study the seasonal variation of these mussels diet and to evaluate if discriminate different geographical origins it is possible or not using their eukaryotic microbiome.

5. Conclusions

Mussels' aquaculture is one of the most important farm cultures, leaded by *M. galloprovincialis* and particularly in Galician area, standing out its DOP that identifies it as sustainable and high quality product (Azpeitia et al., 2017). However, this species is present in several geographic areas and the control of its traceability is complicated. DNA-based methodologies, and NGS technologies offer a good opportunity to develop methods to ensure the traceability and an efficient labeling control. Here a new protocol is selected to study the diversity of the eukaryotic microbiome associated with digestive gland of *M. galloprovincialis*. As part of this protocol, two newly designed primers were tested to improve the recovery of eukaryotic microbiome species without interferences of the mussel DNA. The efficiency of these primers in reducing the amplification of mussel DNA to practically zero and increasing the number of sequences recovered from other eukaryotes present in the sample was corroborated. This protocol will facilitate subsequent studies to determine the seasonal variation of the eukaryotic microbiome in the digestive gland of this species in the areas of interest, as well as the study of potential biomarkers to differentiate individuals bred in that areas from those produced in other geographical areas.

CRediT authorship contribution statement

Amaya Velasco: Writing – review & editing, Investigation, Data curation. **Ren-Shiang Lee:** Writing – review & editing, Resources, Methodology, Investigation. **Marta Muñoz-Colmenero:** Writing – original draft, Visualization, Validation, Supervision, Software, Methodology, Investigation, Formal analysis, Data curation. **Graciela Ramilo-Fernandez:** Writing – review & editing, Visualization, Investigation, Data curation. **Carmen G. Sotelo:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Angeles Longa:** Writing – review & editing, Resources.

Declaration of Competing Interest

Author's declaration indicates no conflict of interest for this manuscript.

Data availability

Data will be made available on request.

Acknowledgements

We would like to thank Marta Pérez Testa and Begoña Correa García for technical assistance. We should also thank to " Centro de Supercomputacion de Galicia" (CESGA; https://www.cesga.es/) for the use of its servers and bioinformatic tools available to the user. This work was carried out with funding from the EU Interreg Atlantic Area Programme (ref. EAPA_87/2016), project "SEATRACES" (https://www.seatraces.eu/), number and with funding from Spanish Ministry of Science and Innovation (ref. PID2020-118012RB-C21), project SEAFOOD-ID project (https://seafood-id.es/).

Data availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aqrep.2024.102031.

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