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SHORT COMMUNICATION

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Water microbiota is not affected by stocking density of the yellowtail kingfish (*Seriola lalandi*) in a recirculating aquaculture system

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Aquaculture of the yellowtail kingfish (*Seriola lalandi*) has great potential considering the closed life cycle of the specimen, its rapid growth rate to market size and high marketability (Abbink et al., 2012). However, these are benthopelagic fish in coastal and oceanic waters and therefore require strictly controlled conditions to be farmed under indoor conditions (Ottolenghi, Silvestri, Giordano, Lovatelli, & New, 2004). In this regard, water quality is one of the most important requirements to take care of, because the accumulation of nitrogenous, sulphurous and phosphorous compounds can affect not only directly but also indirectly the physiological performance of fish and its predisposition to infections (Lim & Webster, 2001). Furthermore, stressful conditions such as stocking density can trigger the oxygen consumption, ammonia excretion, mucus production, feed conversion ratio decrease and other responses causing the deterioration of water quality conditions.

The importance of bacterial communities thriving in the culturing water is becoming a focus of attention for scientists because these can dictate some of the physicochemical conditions of the water while protecting the system from the proliferation of undesirable microorganisms (Huang et al., 2018; Martínez-Córdova, Emerenciano, Miranda-Baeza, & Martínez-Porchas, 2015). For example, *Vibrio harveyi*, an opportunistic fish pathogen frequently implicated in the death of aquatic animals (Stephens & Savage, 2010), may undergo to profound morphological and/or physiological changes when environmental conditions become unfavourable (e.g. limitation of nutrients, low temperature, low salinity, among other; Montánchez et al., 2019). In this regard, Rud et al. (2017) argued that the microbiota in commercial-scale recirculating and semi-closed aquaculture systems rearing Atlantic salmon 'can potentially have a great impact on the robustness and health of the fish'. Garcia-Mendoza, Cáceres-Martínez, Vásquez-Yeomans, and Cruz-Flores (2019) argued that the bacterial community contributed to the maintenance of a balance of the recirculation system of the yellowtail kingfish, preventing the development of infectious diseases; however, this approach contemplated targeted 16S rRNA gene amplification of bacteria cultured in trypticase soy agar supplemented with 2% NaCl. Detailed reports derived from DNA shotgun sequencing of bacterial communities thriving in the culturing systems of the yellowtail kingfish are still absent.

High-throughput sequencing of environmental DNA or shotgun metagenomics is a revolutionary tool that provides the deepest approach known so far for monitoring the taxonomic and functional profiles of microbial communities thriving in any niche (Martínez-Porchas & Vargas-Albores, 2017). Therefore, the aim of this work was to monitor the taxonomic and functional features of microbial communities through high-throughput sequencing of DNA collected from the culturing water of the yellowtail kingfish at two different stock densities in a commercial recirculating aquaculture system in Baja California, Mexico, where marine fish farms are rearing these specimens.

Yellowtail kingfish specimens weighing 9.8 (±1.2) kg and sizing 90.4 (±5.2) cm were stocked at 252 kg (low density, 3.6 kg/m^3) and 424 kg (high density, 6.0 kg/m^3) in two circular tanks with an operative volume of 70 m^3, taking into account that broodstock densities between 3 and 5 kg/m^3 are recommended for inducing sex maturation in captivity (Whatmore et al., 2013). The system was connected to a recirculating aquaculture system (RAS) consisting of a mechanical filter, a foam fractionator, a biological filter,

an ultraviolet light section and an ozonator. This system allows the removal of biological particles, ammonia and nitrite residues, disinfection and aeration/oxygenation, thus decreasing the need for continuous exchange of freshwater and clean water but keeping a healthy environment for fish. Daily water exchange of 20% was performed to maintain the physicochemical parameters of water (oxygen, temperature, pH and nitrogen compounds) within the optimum range for the species (Orellana, Waller, & Wecker, 2014). Fish were fed every two days, with 93% fresh diet (sardine and squid) and 7% formulated feed (Vitalis Cal and Repro, Skretting®) supplemented with vitamin caps.

Water quality parameters remained within optimal intervals: DO 7.75 (±0.02) versus 8.01 (±0.02) mg/L ($p < .05$), temperature 17.9 (±0.1) versus 18.1 (±0.1) °C, pH 7.27–7.29 versus 7.39–7.41, TAN 0.15 (±0.01) versus 0.34 (±0.27) mg/L ($p < .05$), NO_3 14.7 (±1.3) versus 26.6 (±2.2) mg/L, NO_2 0.03 (±0.00) versus 0.14 (±0.01) mg/L ($p < .05$) and alkalinity 193 (±0.86) versus 187.8(±1.00) for the low- and high-density stocking respectively.

For monitoring the microbial communities, samples were randomly collected, considering two replicates per tank. Briefly, 200 ml of the collected water was subsequently filtered through polycarbonate membranes of 3- and 0.2-μm pore size and a

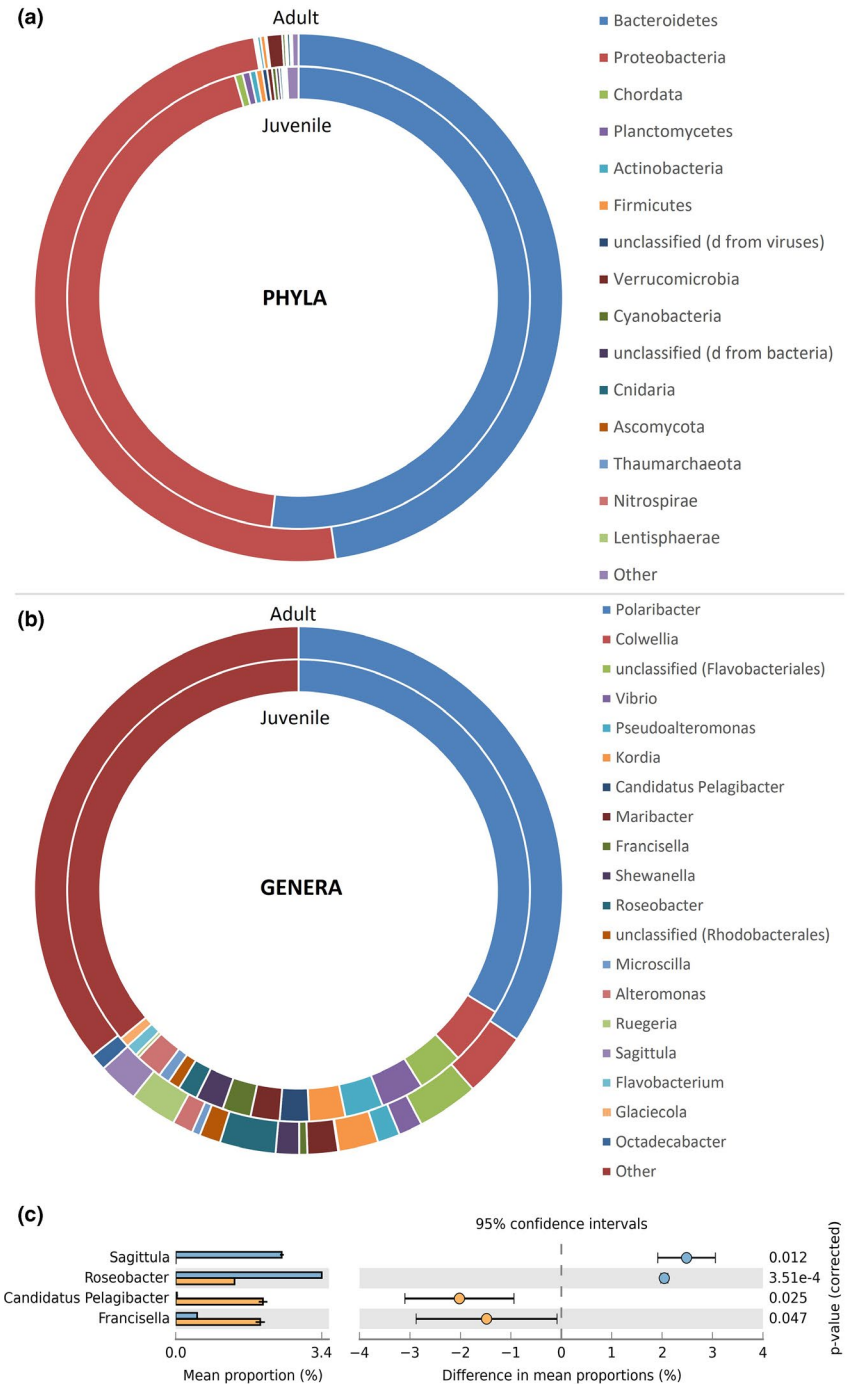

FIGURE 1 Relative abundance of diverse phyla (a) and genera (b) detected in water containing yellowtail kingfish (*Seriola lalandi*) stocked and low and high density. Letter (c) indicates the only genera registering significant differences between low- and high-density tanks, where the blue bars correspond to low density and yellow ones to high density [Colour figure can be viewed at wileyonlinelibrary.com]

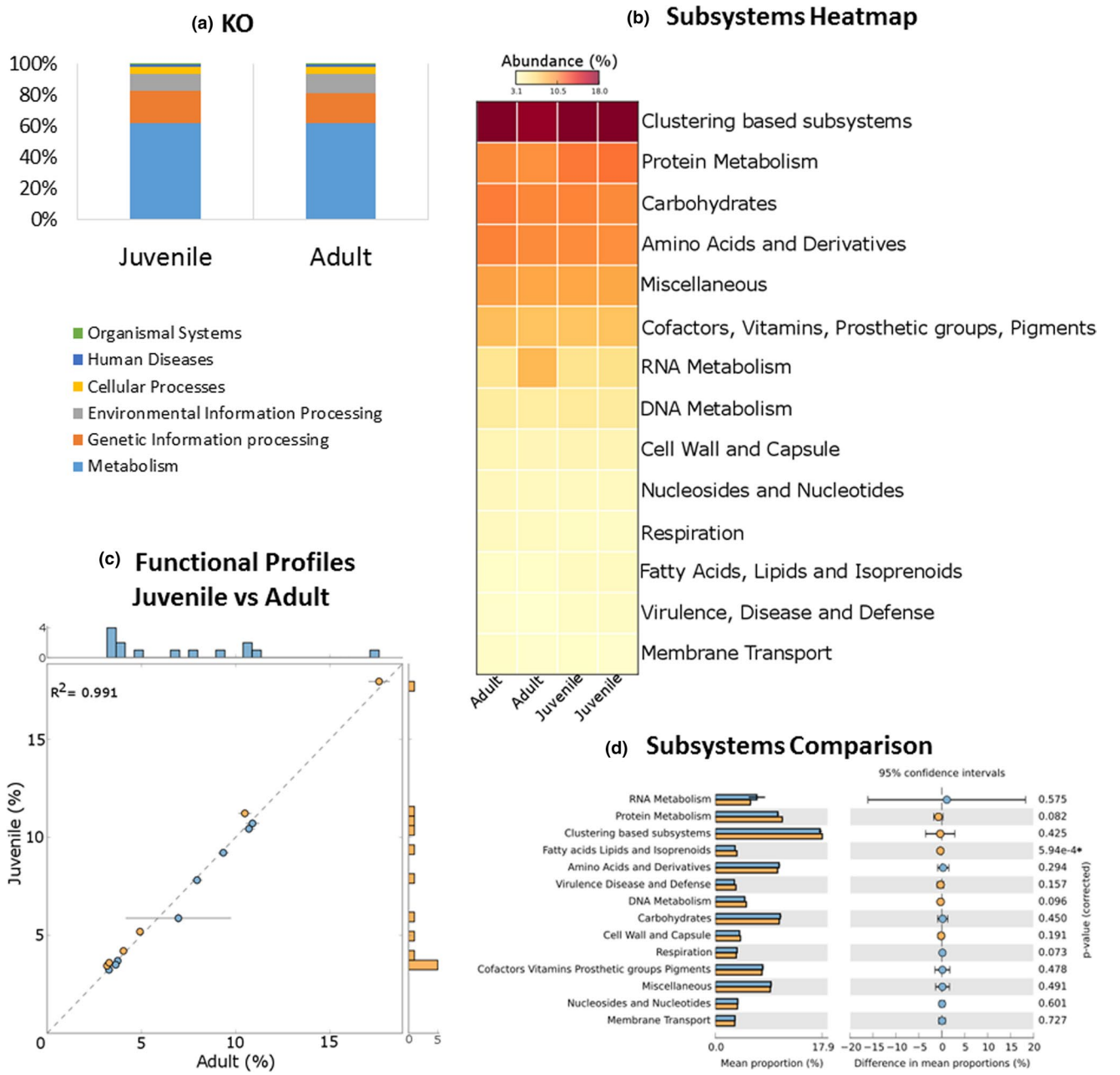

FIGURE 2 Functional profiles of the microbiota detected in the water of tanks containing yellowtail kingfish (*Seriola lalandi*) stocked and low and high density. (a) Heatmap showing subsystems abundance detected in the microbiomes of low- and high-density tanks. (b) Comparison scatterplot considering the abundance of each subsystem, and showing the determination coefficient (R^2), axis bars indicate the abundance of each detected subsystem. (c) Statistical comparison of individual subsystems abundance between low- and high-density tanks. Two metagenomic replicates per density were considered [Colour figure can be viewed at wileyonlinelibrary.com]

diameter of 25 mm (Whatman). Afterwards, DNA was extracted from the filters containing the 3–0.2 μm fraction by using a commercial kit (Wizard® Genomic DNA Purification Kit, Promega). Thereafter, the metagenomic DNA libraries were prepared following the instructions of the Nextera XT DNA Library Preparation Kit (Illumina), and their sequencing was performed at the CICESE facilities under a paired-end mode, using a MiSeq (Illumina) platform. The chemistry used was MiSeq Reagent Kit v3, yielding 150-bp fragments (2x 75 bp).

Raw data sequences in *fastq* format were uploaded to the MG-RAST server (Meyer et al., 2008) for further analysis, using RefSeq database for taxonomic assignments and three databases for functional annotation (i.e. COG, NOG and KO). In this server, the sequences were subjected to quality control including de-replication, ambiguous base filtering (removing sequences with > 5 ambiguous base pairs) and a length filtering (removing sequences with a length of > 2 standard deviations from the mean). Descriptive and comparative statistics such as ANOVA and R^2 were performed using

the STAMP-Bioinformatics software (Parks, Tyson, Hugenholtz, & Beiko, 2014) establishing confidence intervals of ≥ 0.95. Metagenomic sequences were uploaded to the SRA NCBI database under the BioProject: PRJNA625758. They also can be found on the MG-RAST repository (ID number mgp91491).

Averages of 2,246,316 and 2,719,401 sequences with predicted features were obtained after quality control steps for the water of the low- and high-density tanks respectively. The α-diversity based on the distribution of the species-level annotations resulted in 153 and 124 species in low- and high-density tanks respectively. However, similar taxonomic structures and functional profiles were observed between both microbiomes registering a determination coefficient (R^2) of 0.99. Proteobacteria and Bacteroidetes accounted for 95.6 and 97.3% of the reads assigned to this taxonomic level in both stock densities (Figure 1a), followed by other taxonomic groups that individually registered relative abundances of ≤ 0.55%. Recent studies about the bacterial structure and function of the global ocean microbiome revealed that all these phyla constitute the main core of the taxonomic profile regardless of the geographical zone (Sunagawa et al., 2015). The Eukarya domain was also represented by the presence of phyla Chordata, Cnidaria and Ascomycota.

Similar results were registered at the genus level (R^2 = 0.98), where 60%–62% of the abundance in both yellowfish tanks was dominated by 15 genera, including *Polaribacter* (33.8%–34.5%), *Colwellia* (3.9%–4.1%), bacteria derived from Flavobacteriales (3.1%–3.7%), *Vibrio* (1.5%–3.0%), *Pseudoalteromonas* (1.4%–2.7%), *Kordia* (2.4%–2.5%), *Candidatus* Pelagibacter (0.1%–2.0%), *Maribacter* (1.9%–2.0%), *Francisella* (0.5%–1.98%), *Shewanella* (1.4%–1.9%), *Roseobacter* (1.4%–3.4%) and others registering at least 1% in abundance (Figure 1b). However, the abundance of four of these 15 genera shown significant differences between yellowfish densities (Figure 1c).

The research performed by Garcia-Mendoza et al. (2019) revealed that the phylum Proteobacteria was predominant in the culturing water of the yellowtail kingfish, including the orders Vibrionales and Alteromonadales (≈60%), whereas Bacteroidetes was the most dominant phylum detected in our study. This is because the approach used contemplated the metagenomics DNA present in water, whereas the former approach considered cultivated bacteria only. These results suggest that only a small fraction of the bacterial diversity thriving in these systems was known.

Among the most abundant bacterial groups, *Polaribacter* is a heterotrophic Gram-negative, aerobic bacteria genus belonging to the family Flavobacteriaceae. The high abundance of this genus has been detected in different parts of RAS such as compartments and biofilters (Martins et al., 2013; Ruan, Guo, Ye, Liu, & Zhu, 2015). Although this genus has been recognized as a potential pathogen, studies of Bacteroidetes phylum across the North Atlantic Ocean have demonstrated that *Polaribacter* is a dominant genus in these marine waters (Gomez-Pereira et al., 2010). Most of the other genera detected in this study have also been reported in fish RAS and their biofilters (Ruan et al., 2015; Schreier, Mirzoyan, & Saito, 2010), but only two of them (*Pseudoalteromonas* and *Shewanella*) have been reported as regular and abundant members of the yellowtail kingfish gut microbiota (Ramírez & Romero, 2017). Indeed, *Pseudoalteromonas* sp. has been successfully used as a probiotic supplement on the larval culturing of *S. lalandi*, improving survival rates (Leyton et al., 2017). In this latter study, the probiotics were supplied indirectly through zooplankton enrichment in the diet; however, our results demonstrate that *Pseudoalteromonas* sp. can thrive in water, opening the possibility to use them directly in water.

Regarding functional annotations, highly similar profiles were also observed between low- and high-density tanks. Most of the annotations were associated with metabolism (61.1%–61.6%), genetic information processing (19.5%–21.1%) and environmental information processing (10.4%–12.4%) (Figure 2a). In addition, the functional analysis revealed that clustering-based subsystems, protein and carbohydrate metabolisms were the most abundant (Figure 2b). The overall comparison between functional profiles from low- and high-density tanks resulted in an R^2 = 0.991 (Figure 2c), supporting the notion that there is not much difference between microbiotas in the culturing water of the yellowtail kingfish stocked at different densities. Nevertheless, one of these subsystems resulted to be statistically different between microbiomes (fatty acids, lipids and isoprenoids; Figure 2d). Taken together, the overall results were expected considering the high similarity of taxonomic profiles between microbiotas. Therefore, we conclude that the microbiota thriving in water is not affected by the stocking density of fish, but rather is maintained relatively stable probably because of the action of the recirculation system.

AUTHOR CONTRIBUTIONS

M. Martínez Porchas wrote the manuscript and contributed to data analysis. F. Lafarga-De la Cruz designed the study, collected the samples and revised the draft manuscript. F. Aguilera analysed the data and revised the manuscript. F. Cicala analysed the data. A. Lago-Lestón designed the study, analysed data and revised and corrected the draft manuscript.

DATA AVAILABILITY STATEMENT

Metagenomic data used in this study are available at SRA NCBI and the MG-RAST public repositories under BioProject PRJNA625758 and the ID project *mgp91491* respectively.

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