Characterization of the skin and gill microbiomes of the farmed seabass (*Dicentrarchus labrax*) and seabream (*Sparus aurata*)

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Abstract

There is substantial evidence showing that the microbiome of teleosts plays a key role in host health and wellbeing. Aquaculture practices increase the risk of dysbiosis (i.e. microbial imbalance), which is known to facilitate pathogen infections. The skin and gills are the primary defense organs against pathogens, thus, characterizing their microbiome composition in farmed fish is pivotal for detecting potential alterations that may lead to disease susceptibility. Here, we assessed the skin and gill microbiomes of two of the most important adult fish species farmed in southern Europe, the seabass and the seabream, during winter months. We coupled next-generation sequencing (MiSeq) of the 16S rRNA V4 region with the DADA2 bioinformatic pipeline to assess microbial composition and structure. Variation in microbial alpha-diversity (intra-sample) and taxa proportions were assessed using analysis of variance. Differences in beta-diversity (between-sample) were tested using permutational multivariate analysis of variance. Microbiomes of both tissues (n=30 per species) identified 19 bacteria phyla, dominated by the phyla *Proteobacteria* (44 - 68%) and *Bacteroidetes* (15 - 37%); the families *Flavobacteriaceae* (11 - 28%), *Rhodobacteraeae* (4 - 8%) and *Vibrionaceae* (2 - 17%); and the genera *Rubritalea* (4 - 13%), *Pseudomonas* (4 - 8%) and the NS3a marine group (4 - 12%). Mean relative proportion of these taxa, some alpha-diversity indices and all beta-diversity distances varied significantly between tissues within and between species. ASVs belonging to the genera *Polaribacter* and *Vibrio*, which include several species that are pathogenic, were detected in the core microbiomes of seabass or seabream.

Keywords: aquaculture, 16S rRNA, microbiome, pathogens, fish farm

1 Introduction

Seminal studies conducted in mammals established a link between the microbiome and the host's innate immune response, with implications for host health and wellbeing (see for example reviews by Belkaid and Hand, 2014; Lynch and Peterson, 2016; Nelson et al., 2014). Furthermore, differences in microbial composition can account for differential disease susceptibility in humans (e.g. Börnigen et al., 2013; Dunn et al., 2016; Pérez-Losada et al., 2015; Pérez-Losada et al., 2018) and teleosts (reviewed by Kelly and Salinas, 2017). Particularly problematic for fish, pathogenic bacteria that naturally reside in the aquatic environment can also form part of their microbiomes (e.g. Borchardt et al., 2003; Califano et al., 2017; Rivas et al., 2011; Rud et al., 2017) and cause disease if there is a shift in abundance (i.e., dysbiosis) (e.g. Hess et al., 2015). While the skin microbiome of unstressed fish is dominated by taxa known for their probiotic and antimicrobial activity, the microbiome of stressed fish is dominated by potential pathogens (see Boutin et al., 2013). Although mucosal surfaces, such as skin, gills and the gut, do act as primary barriers to disease (reviewed by Gómez and Balcázar, 2007), they can be affected by several pathogens (e.g. *Aeromonas septicemia*, see Balebona et al., 1998; Doukas et al., 1998), which may cause significant losses. Aquaculture practices also impact microbial communities in the epidermal mucosa of fish. Overcrowding and low oxygen concentrations, typical in fish farms, result in host stress and induce dysbiosis in the skin microbiome, facilitating the proliferation of opportunistic pathogens (e.g. Boutin et al., 2013). At the same time, infectious diseases that frequently affect farmed fish can also induce dysbiosis, generally favouring increased abundance of opportunistic bacteria creating complex feedback mechanisms (e.g. Llewellyn et al., 2017; Reid et al., 2017).

Seabass (*Dicentrarchus labrax*) and seabream (*Sparus aurata*) are the two most important fish species farmed in southern Europe; their productivity, however, is greatly affected by infectious diseases, which can account for losses of 15% to 40%, respectively (Lane et al., 2014). Given the role skin and gill microbiomes play in fish innate immunity (Gourzioti et al., 2016; Pellizzari et al., 2013) and the economic impact of diseases in fish aquaculture, characterizing the microbiomes of these two fishes is paramount. Additionally, anthropogenic stressors (e.g. rise of sea temperature and pollution) and farming conditions (e.g. high densities) aggravate bacterial diseases causing external lesions in skin and gills (e.g. photobacteriosis and vibriosis) of farmed seabass and seabream (e.g. Avendaño-Herrera et al, 2006; Bakopoulos et al., 2018; Frans et al., 2011; Gourzioti et al., 2016; Pellizzari et al., 2013; Weber et al., 2010). To this end, identifying potential fish pathogens could help to design more efficient prevention and treatment strategies. The first assessment of the skin microbiome of adult seabass and seabream showed that inter-individual variability was comparable to interspecific variability (Chiarello et al., 2015). Recently, Tapia-Paniagua et al. (2018) found a reduction in beneficial bacteria from the skin microbiomes of ulcered compared to healthy seabream. Differences in microbiome diversity of the skin of seabass have also been assessed in three different fish farms located in Ria de Aveiro, northern Portugal (Pimentel et al., 2017). Despite high inter-individual variation, microbial composition was found to act as a unique signature of each

individual's geographic origin (Pimentel et al., 2017). Although the authors controlled for ontogenetic effects, which affect skin microbiome (e.g. Sylvain and Derome, 2017), they acknowledged that other factors, such as different farming practices and probiotic use, may have explained some of the observed differences (Pimentel et al., 2017). Moreover, previous disease history (e.g. Llewellyn et al., 2017; Tapia-Paniagua et al., 2018; Reid et al., 2017) and host physiology (e.g. Apprill et al., 2014) may have influenced the composition of fish skin microbiomes. Other key factor impacting microbial composition differences between groups is sample size and longitudinal (time) variation (Knight et al., 2018). Not surprisingly, skin and gill microbiome composition of seabass and seabream varied greatly between previous studies, as they have been cross-sectional (one time point) and included few individuals (Chiarello et al., 2015; Pimentel et al., 2017; Tapia-Paniagua et al., 2018).

In the present study, we monitored the microbiome composition and structure of the skin and gills of 30 seabass and 30 seabream healthy adults over winter (December to February) using 16S rRNA next-generation sequencing (MiSeq). Our main aims here were to characterize the baseline diversity of the skin and gill microbiomes of these two farmed species and identify potential pathogens or opportunistic bacteria.

2 Material and Methods

2.1 Sample collection and preparation

Thirty individuals of both seabass and seabream were collected in 19 of December 2016, 16 of January 2017 and 13 of February 2017 (10 specimens of each species per month) from a commercial fish farm located in an estuarine environment, the Ria Formosa (Portimão), southern Portugal. Seabass and seabream sampled were about 2 years old and individuals weighted on average, 384 g and 318 g. The fish were reared in two separate ponds, at a density of ca. 4.4 kg/m^3 (ca. 130 individual seabass) and 5.2 kg/m^3 (ca. 150 individual seabream), with the same open water circulation systems, thus subjected to the same environmental conditions. The mean water temperature 30 days before each sampling point was 16.6 ºC, 15.3 ºC and 14.4 ºC, and the photoperiod for each sampling point was 9 h 35 min, 9 h 54 min and 10 h 45 min, respectively. All fish were fed with the same commercial feed and they shared the same clinical history. All fish were considered healthy, with no external lesions and no pathologies detected during the sampling period. Individuals were randomly caught from each tank using a fishing pole, and skin and gill swabs were collected using tubed sterile dry swabs (Medical Wire & Equipment, UK). Skin samples were taken by swabbing several times along the right upper lateral part of the fish from head to tail; gill swabs were taken from the right filaments between the first and second arch. Swabs were immediately stored at -20ºC until transported on dry ice to the CIBIO laboratory by airmail where they were kept at -80ºC until processing. DNA from a total of 120 samples (60 skin and 60 gills) was extracted using the PowerSoil DNA Isolation Kit (QIAGEN, Netherlands), following the manufacturer's protocol. DNA concentration was measured with the NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, USA) and extractions were sent on dry ice by airmail

to the University of Michigan Medical School (USA) for amplification and sequencing according to the protocol of Kozich et al. (2013). Each sample was amplified for the V4 hypervariable region of the 16S rRNA gene (~250 bp), which has been widely used to characterize microbiomes from vertebrates (Earth Microbiome Project, Gilbert et al., 2014), including fish (e.g. Carlson et al., 2017; Llewellyn et al., 2015; Nielsen et al., 2017; Wang et al., 2017). Amplicon libraries were sequenced in a single run of the Illumina MiSeq sequencing platform.

2.2 Data and statistical analyses

Raw FASTQ files were analyzed using the Quantitative Insights Into Microbial Ecology 2 (QIIME2; release 2018.4) platform. Clean sequences were aligned against the SILVA (132 release) reference database (Quast et al., 2012) with DADA2 pipeline (Callahan et al., 2016). Samples were rarefied to the minimum read count (9,087) and a feature table containing amplicon sequence variants (ASVs) was constructed. ASVs with less than 0.01% of reads across samples were eliminated (Nelson et al., 2014). The core microbiome was assessed for the skin and gill of seabass and seabream, separately. An ASV was considered as part of the core microbiome if present in 100% of samples in each group. Rarefaction curves were performed to examine sampling depth (Supplementary Figure 1).

Microbial taxonomic alpha-diversity (intra-sample) was calculated using Shannon, ACE, Fisher and Faith's phylogenetic diversity (PD) indices as implemented in the R package phyloseq (McMurdie and Holmes, 2013). Species beta-diversity (inter-sample) was estimated using phylogenetic Unifrac (unweighted and weighted) and Bray-Curtis distances. Dissimilarity between samples was assessed by principal coordinates analysis (PCoA). Variation in microbial alpha-diversity and taxa composition were assessed using one-way analysis of variance (ANOVA). Differences in community composition (beta-diversity) were tested using permutational multivariate analysis of variance for Unweighted and Weighted Unifrac and Bray-Curtis indices with 1,000 permutations, as implemented in the adonis function of the R vegan package. In our microbiome statistical analyses we compared i) tissues within each fish species (skin x gills) and ii) fish species within each tissue (seabass x seabream) – see Table 2. We used the three sampling months (December to February) as temporal replicates, rendering a total of 30 microbiome samples per comparison per tissue. All analyses were performed in R studio v1.0.143 (Studio R, 2012).

3 Results

3.1 Taxonomic bacterial composition and core microbiome of seabass and seabream

Approximately 3.2 million raw reads were retrieved (1.7 million for seabass and 1.5 million for seabream) and the number of sequences per sample ranged between 9,087 and 3,537,652. These sequences corresponded to 8,136 unique ASVs, from which ASVs with less than 0.01% of sequences across all samples and ASVs belonging to Archaea were removed, resulting in 556 unique ASVs and 3,246,429 sequences. Of the 457 ASVs found in the skin of the seabass, only

24 were common to all individuals sampled, thus forming the core microbiome (Table 1). Of the 466 ASVs found on the gills of the seabass, only 7 were shared among all individuals. The same pattern was observed in the seabream, where 15 out of 532 skin ASVs and 2 out of 539 gill ASVs were present in all individuals (Table 1). These results highlight the high inter-individual variability found in both tissues, especially the gills (Table 1, Figures 1 and 2).

Of the total 19 bacteria phyla identified across all samples, *Proteobacteria* and *Bacteroidetes* were the most abundant in both tissues (Figure 1, Table 1). ASVs from four (*Proteobacteria*, *Bacteroidetes*, *Chlamydiae* and *Verrucomicrobia*) of these 19 phyla formed part of the core microbiome (Figure 2). Moreover, the phyla *Dependentiae* (0.2% of ASVs and sequences) and *Patescibacteria* (0.2% of ASV, 0.1% of sequences) were unique to the microbiome of seabream, while the phyla *Spirochaetes* (0.2% of ASVs and 0.1% of sequences) was unique to the gill microbiome of seabass.

The phyla *Proteobacteria* and *Bacteroidetes* accounted for 69% to 72% of all ASVs and 62% to 87% of all sequences in both species and for 50% to 93% of all phyla in the core microbiomes (Figure 2, Table 1). It was possible to identify 106 families, from which ASVs belonging to 16 families formed the core microbiome of both species. Altogether, *Flavobacteriaceae* (*Bacteroidetes*), *Rhodobacteraeae* (*Proteobacteria*) and *Vibrionaceae* (*Proteobacteria*) accounted for 19% to 21% of ASVs, 17% to 51% of sequences, and 29% to 50% of all families in the core microbiome of both tissues (Figure 2, Table 1). From the 117 genera identified, ASVs belonging to 16 of these genera formed the core microbiome of both species. The NS3a marine group (4% - 12%), *Rubritalea* (4% - 13%) and *Pseudomonas* (4% - 8%) were the most abundant genera in the skin and gill of both species (Table 1). *Polaribacter* (7% - 50%) was highly abundant in both tissues, and *Polynucleobacter* (14%) and *Vibrio* (7%) were highly abundant in the gill of seabass and in the skin of seabream, respectively (Figure 2, Table 1).

3.3 Microbial diversity

When comparing the alpha-diversity of bacteria between tissues within each species, significant differences were detected between the skin and gills of seabass (ANOVA, $P < 0.05$; Table 2, Figure 3), but not for seabream (ANOVA, $P > 0.05$; Table 2, Figure 3). The alpha-diversity of the skin microbiome was significantly different between the seabass and the seabream for all indexes (ANOVA, $P < 0.05$; Table 2), except the Shannon index (ANOVA, $P = 0.4$; Table 2). On the other hand, the gill microbiomes were similar between species for all indices except PD (ANOVA, $P = 0.03$; Table 2).

Analysis of the PCoA shows that species and tissues within species cluster separately and that there is a higher variation in the gill microbiomes when compared to the skin (Figure 4). There were significant differences in beta-diversity estimates between tissues within each species and between tissues across species (Adonis, $P = 9.9^{-5}$ for all; Table 2).

Mean proportions of bacterial taxa varied between the two fish species and tissues (Table 2). In the seabass, the abundance of *Bacteroidetes*, *Flavobacteriaceae*, NS3a marine group, *Rubritalea*, *Pseudomonas*, *Polaribacter* and *Polynucleobacter* were significantly different between the skin and gill (ANOVA, $P < 0.05$, Table 2). In the seabream, the mean proportion of *Proteobacteria*, *Bacteroidetes*, *Flavobacteriaceae*, *Rhodobacteriaceae*, *Vibrionaceae*, NS3a marine group, *Polaribacter*, *Polynucleobacter* and *Vibrio* varied significantly (ANOVA, $P < 0.05$) between the skin and gill microbiomes (Table 2). Finally, *Proteobacteria*, *Vibrionaceae*, NS3a marine group, *Pseudomonas*, *Polaribacter*, *Polynucleobacter*, and *Vibrio* varied significantly between the skin microbiomes of seabass and seabream (ANOVA, $P < 0.05$, Table 2), while *Proteobacteria*, *Rhodobacteriaceae*, *Vibrionaceae*, NS3a marine group, *Rubritalea*, *Polaribacter* and *Polynucleobacter* varied significantly between their gill microbiomes (ANOVA, $P < 0.05$, Table 2).

4 Discussion

Characterizing the microbiome composition and structure of the mucosal surfaces of economically important fish species, such as the seabass and the seabream, is of paramount importance in order to detect imbalances and prevent potential disease outbreaks in fish farms. Here, we showed significant differences in both the composition and structure of the microbial communities residing in the skin and gills of seabass and seabream, which is in line with previous findings of both fish species (Chiarello et al., 2015). The skin microbiomes were found to be species-specific as in other fish species (e.g., the stripped mullet, red snapper, spotted seatrout, sand seatrout, pinfish and Atlantic croaker; Larsen et al., 2013). Despite the high inter-individual variation, overall, the seabream microbiomes were less diverse than those of the seabass (Figure 3).

4.1 Core microbiome composition

Proteobacteria (50 - 60%) and *Bacteroidetes* (29 - 50%, Table 1) formed the main components of the skin and gill microbiomes of seabass and seabream. *Proteobacteria* is the most common phylum reported in the skin and gill microbiomes of teleosts (see for example the review by Llewellyn et al., 2014), including the skin microbiome of seabass and seabream (Chiarello et al., 2015; Pimentel et al., 2017; Tapia-Paniagua et al., 2018). A predominance of the phylum *Bacteroidetes* has also been previously reported in seabass and seabream (Chiarello et al., 2015; Tapia-Paniagua et al., 2018), as well as in the skin of many other fishes, such as in the brook char (Boutin et al., 2014), rainbow trout (Lowrey et al., 2015), channel catfish (Larsen et al., 2014), tambaqui (Sylvain et al., 2016), among others (see Doane et al., 2017; Larsen et al., 2013; Larsen et al., 2015; Legrand et al., 2018; Leonard et al., 2014). The gill microbiome of the bluefin tuna (Valdenegro-Vega et al., 2013), rainbow trout (Lowrey et al., 2015) and yellowtail kingfish (Legrand et al., 2018) were also found to be dominated by *Bacteroidetes*.

In the present study, from the 16 genera identified in the core microbiome of the skin and gill of adult seabass and seabream, the highest percentage of amplicon sequence variants (ASVs) belonged to the NS3a marine group, *Rubritalea* and *Pseudomonas* genera. Besides these three, the microbiome of seabass also exhibited an elevated abundance of the genera *Polaribacter*, *Polynucleobacter* and *Arcobacter*; while the microbiome of seabream included high abundance of *Polaribacter* and *Vibrio*. The genus *Pseudomonas* has been previously reported to be highly represented in the skin microbiome of seabass (Pimentel et al., 2017), cod (Wilson et al., 2008), mosquitofish (Leonard et al., 2014), gulf killifish (Larsen et al., 2015) and others (see Colwell and Liston, 1962; Horsley, 1973; Horsley, 1977; Larsen et al., 2013). However, we found some differences in microbial composition at the genus level in comparison with previous studies of seabass and seabream; Tapia-Paniagua et al. (2018), for example, found *Staphylococcus* and *Lactobacillus* to be the most abundant in the skin microbiome of seabream. This in not unexpected since, the skin microbiome of seabass comprises genera that are unique signatures of specific earth growth ponds, even though these ponds were geographically close (Pimentel et al., 2017). Besides spatial variation in fish location, environmental conditions (such as water temperatures and water supply [e.g. Lokesh and Kiron, 2016; Tapia-Paniagua et al., 2018]), host physiology and even clinical history (Apprill et al., 2014; Llewellyn et al., 2017) could contribute to explain the observed differences.

Ontogenetic shifts in microbiome composition have been described in several fish species (e.g. Atlantic salmon, Llewellyn et al., 2015; Zarkasi et al., 2014; Zebrafish, Stephens et al., 2016; discus, Sylvain and Derome, 2017). The larval microbiome tends to reflect more the microbial community of the surrounding water (Stephens at al., 2016; Sylvain and Derome, 2017), while adult fish harbour a more adapted and stable microbial community (e.g. Llewellyn et al., 2015). Califano et al. (2017) even reported an increase in the microbiome composition of seabream larvae between day 2 and day 34. This pattern, however, is far from being universal as decreased diversity with age has been reported in other fish species (Stephens et al., 2016; Yan et al., 2016).

As with most microbiome research, it is important to note any methodological differences that might explain variation. One of such methodological differences relates to our skin sampling method; Chiarello et al. (2015) used tissue from different fins, while in the present study and in Tapia-Paniagua et al. (2018) and Pimentel et al. (2017) we targeted skin mucous. Lowrey et al. (2015) uncovered high diversity of bacteria in the different dermal layers of skin, suggesting that mucosal diversity is an underestimation of the actual skin microbial diversity. Moreover, specifically for this study, the sequenced 16S variable region and the sequencing platform might have impacted taxonomic assignment. We sequenced the V4 region by synthesis (MiSeq), while Chiarello et al. (2015), Pimentel et al. (2017) and Tapia-Paniagua et al. (2018) used different combinations of other 16S regions and sequencing platforms (pyrosequencing and sequencing by synthesis). While differences in outputs provided by different sequencing methods are widely acknowledged (e.g. Frey et al., 2014; Li et al., 2014), debate regarding the most appropriate region for microbiome studies is still ongoing (e.g.

Guo et al., 2013; Mizrahi-Man et al., 2013). Finally, these results are likely to have been affected by the different analytical pipelines used to analyze the sequence data - amplicon sequence variants (ASVs) in this study versus Operational Taxonomic Units (OTUs) in previous studies.

4.2 Potential pathogens detected in the core microbiomes

Several ASVs belonging to genera comprising opportunistic and potential pathogenic bacteria were recovered from the skin and gill core microbiomes of apparently healthy individuals of seabass and seabream. *Polaribacter* is one such taxa, recovered from the skin and gills of both species (Figure 2, Table 1). This genus is often found in diseased fishes, being considered opportunistic and colonizing already weakened hosts (Bornø and Linaker, 2015). Species from this genus seem to be common in fish farms and have been reported in the water and biofilm of recirculating and semi-closed aquaculture systems rearing Atlantic salmon, turbot and the Senegalese sole (Martins et al., 2013; Rud et al., 2017). The genus *Vibrio*, present in the gills of seabass and in both the gills and skin core microbiomes of seabream, harbours species associated with several diseases in these fish and many are considered opportunistic pathogens (e.g. Pujalte et al., 2003a; Weber et al., 2010). *Vibrio anguillarum* and *V. alginolyticus*, for example, cause skin lesions, and *V. splendidus* has been involved in several disease outbreaks (e.g. Frans et al., 2011; Pujalte et al., 2003a). *V. harveyi* is another important pathogen causing tail rot disease in farmed seabream (Austin and Zhang, 2006; Haldar et al., 2010), comprising many strains that are fatal to seabass (Pujalte et al., 2003b). However, due to taxonomic assignment limitations, it was not possible to ascertain to which species these ASVs belonged to and if they are, indeed, pathogenic. If so, the high prevalence of these ASVs means that, in case of dysbiosis, these bacteria may overgrow and impact fish health.

5 Conclusion

The skin and gills of fish are exposed to many pathogens present in the marine and freshwater environment and represent an important barrier preventing pathogen invasion (e.g. Trivedi, 2012). The links between microbiome composition and disease resistance are now well established in mammals and teleosts (e.g. Britton et al., 2014; Gómez and Balcázar 2007; Gomez et al., 2013; Kelly and Salinas, 2017), and alterations in the microbiome often precede the onset of disease (reviewed by Munang'andu et al., 2018). Microbial imbalance, however, is not easily detected unless baseline information regarding microbiome composition and structure are established. Here, we describe the skin and gills microbiomes of farmed healthy seabass and seabream adults through three sampling months, thus yielding a more robust assessment of the microbiome of these two species. Our results show that seabass and seabream host different microbiomes despite sharing the same environment. Furthermore, high levels of intra- and inter-individual variability were found across tissues. Additionally, several potential pathogens were detected in the core microbiome of both species, which could lead to potential disease outbreaks during dysbiosis.

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