Identification, phenotypic characterization and selection of gilthead seabream (*Sparus aurata*) associated bacteria for application as putative probiotics in fish larviculture.

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Abstract

Probiotics supplementation is a promising strategy to control pathogens in aquaculture, particularly during larviculture where the fish's immune system is underdeveloped. In this work, bacterial symbionts isolated from gilthead seabream (*Sparus aurata*) eggs, larvae, and juveniles were selected, based on taxonomical and physiological criteria, for their potential use as probiotics, and the best candidates evaluated during a fish larvae rearing trial. The studied symbionts (97 isolates) were classified into 32 genera in the *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* phyla. Thirty-five non-pathogenic (based on current literature) and non-redundant isolates were characterized for hydrolytic enzyme (chitinases, proteases, amylases, lipases, and cellulases) production and antagonistic activity towards bacterial pathogens of fish. The isolates *Phaeobacter inhibens* L23 (best pathogen antagonist) and *Arthrobacter agilis* E13 (versatile producer of hydrolytic enzymes) were selected as co-inoculants of fish eggs and live feed (rotifers) and assessed as probiotics in gilthead seabream larval rearing. Using 16S rRNA gene amplicon sequencing, a decrease in abundance of opportunistic taxa (*e.g.*, *Vibrionaceae*) and increase in abundance of putatively beneficial symbionts (*e.g.*, *Rhodobacteraceae* species) was observed in probiotic-treated rotifers. While no differences in dry weight and length were observed between fish larvae fed probiotics-treated *versus* control rotifers, slightly higher larval survival rates were recorded under probiotic treatment by the end of the rearing trial. These results suggest that modulation of the rotifer-associated microbiome through co-inoculation of the here selected probiotics is feasible, yet further research is needed to improve fish larval wellbeing using rotifers as delivery systems of beneficial bacteria to fish.

Keywords: Antimicrobial activity; Aquaculture; Hydrolytic activity; Microbiome; Rotifers.

Introduction

Worldwide human population is constantly growing, posing increasing pressures on terrestrial and marine natural resources [1-3]. One example is the excessive fish consumption. Fishes are one of the most consumed animals globally and, consequently, wild stocks of marine fish are over-exploited. One strategy, among several measures to confront this natural resources depletion, is the development and optimization of aquaculture techniques [3]. This practice can be performed in three settings: in-land (mainly freshwater), coastal (mainly brackish water) and open sea (mainly marine water) [4-6], but in terms of rearing characteristics, it is directly related for example with the type of reared fish, the facilities available, and economical aspects. The rapid expansion of this industry also created environmental problems, such as seasonal oversupply, disease, pollution (*e.g.*, biological, organic and chemical), genetic deterioration, and other concerns [7-9].

Sparus aurata (gilthead seabream) is one of the most important reared fishes worldwide, especially in Europe, up to almost 7% of the global aquaculture production [10, 11]. Over the last 20 years, its market value has rapidly increased along the Mediterranean basin, with 90% of the seabream production being concentrated in six countries: Turkey (37%), Greece (25%), Egypt (14%), Spain (9%), Tunisia

(4%) and Italy (4%) [12, 13]. One of the major problems in fish rearing (mainly economically) is the existence of pathogens that cause infectious diseases, which can account for losses up to 10% at the end of the seabream on-growing production period [13]. Of all the fish life stages, the larval phases are the ones most affected by these pathogens since if these become infected the consequence might be mortalities of up to 100% [14]. To mitigate some of these challenges, the introduction of beneficial microbes (*e.g.*, probiotics) in aquaculture environments has been considered a highly promising and sustainable strategy to diminish fish diseases and to reduce antibiotic application. The vast majority of these microbes are found in the animals microbiome (total pool of microorganisms, whether beneficial or pathogenic, present in a certain habitat, sample or host tissue, their genomes, interactions and the surrounding environmental conditions) or in the adjacent ecosystem [15]. Although the exact molecular mechanisms and modes of action may not always be fully demonstrated, probiotics (living microbes that improve host health when supplied in adequate amounts [16]) are known to usually enhance the host's non-specific defensive system, through natural processes such as competition for biological surfaces and natural resources [17], and through the production of compounds [18] and enzymes (*e.g.*, proteases, lipases, amylases, cellulases, and chitinases) which can suppress pathogen proliferation or act as host growth promoters [19-25].

No studies of culturable bacteria associated with *S. aurata* early developmental stages have thus far been performed. In this context, the major aim of this work was to select, based on taxonomical and physiological criteria, bacterial symbionts of fish from early developmental stages (eggs, larvae, and juveniles), acquired through a culture-dependent approach, and evaluate the two best complementary (best antagonist and best enzymatic producer) candidates for their potential use as probiotics during a fish larval rearing trial. With this work, we hypothesized whether a modulation of the rotifer-associated microbiome through co-inoculation of two selected probiotics was feasible and whether effects of probiotic-treated rotifers supplementation on larval growth and survival could be observed.

Methodology

Taxonomic identification and phylogenetic analyses of cultivated bacteria: The starting material of this work consisted of 97 bacterial isolates (32 from eggs, 31 from larvae, and 34 from juveniles) previously retrieved by Borges *et al*., (unpublished data). These isolates were here subjected to taxonomic identification and phenotypic characterization. 16S rRNA gene-based taxonomic identification of the isolates was conducted using the Classifier and Sequence Match tools of the Ribosomal Database Project (RDP). In addition, sequences were matched on the NCBI database using the BLASTn algorithm which allows us to identify the closest type-strains to our queries.

Bioactivity screening of cultivated bacterial symbionts of fish: As a result of the taxonomic analyses described above, 45 out of 97 isolates, representative of non-pathogenic (based on current literature) and non-redundant 16S rRNA gene sequences, were subjected to *in vitro* screenings for potentially probiotic properties. The non-redundant isolated bacteria were screened to produce lipase, amylase, cellulase, protease, and chitinase enzymes in agar plate assays. Cross-streak and soft-agar assays were used to examine the isolates' activity against the

fish pathogens *Vibrio parahaemolyticus*, *Photobacterium damselae* subsp. *piscicida*, and *Streptococcus iniae*. For normalization, the enzymatic and antagonistic activity index was calculated [26].

Selection of the two best isolates: After bioactivity screenings assessment, the two strains that best complement each other (*Arthrobacter agilis* E13 and *Phaeobacter inhibens* L23) were selected as the potential probiotics to be used in an *in vivo* fish larval rearing experiment.

Larval rearing trial set-up and sampling: *Sparus aurata* eggs were obtained naturally from brood stock adapted to captivity at Estação Piloto de Piscicultura de Olhão (EPPO), Instituto Português do Mar e da Atmosfera (IPMA) (Olhão, Portugal), designated hereafter as "EPPO-IPMA". The production of live feed (microalgae, rotifers, *Artemia*, and microalgae) for the gilthead seabream (*Sparus aurata*) larval trial was carried out at EPPO-IPMA [27] according to in-house protocols. The *S. aurata* larval trial comprised two treatments (Control and Probiotic treatments) with four replicate tanks each treatment. Half of the eggs were incubated with a potential probiotic mixture (1 x 10^6 cfu/mL both for *A. agilis* E13 and for *P. inhibens* L23) in a volume of 1 L for 1 h. The feeding protocol of the four tanks corresponding to the probiotic treatment included the provision of rotifers enriched with probiotics (1 x 10^6 cfu/mL both for *A. agilis* E13 and for *P. inhibens* L23) whilst the control treatment tanks included the provision of non-enriched rotifers. To evaluate the effect of the isolates' addition to the eggs and the rotifers enriched with a mixture of probiotics on the larval performance, 10 larvae samples per tank were collected at 2, 7, 14, and 35 DAH (Day After Hatching) for biometry (total length and dry weight) analysis. The remainder larvae at 35 DAH were used for survival analysis. Treated rotifer samples were also obtained for further 16S rRNA gene amplicon sequencing.

Total DNA extraction, quantification and PCR amplification from control and probiotic-treated rotifers: The DNeasy® Power Soil® Kit (QIAGEN®, Germany) was used to extract total community DNA (TC-DNA) from rotifers with and without probiotics according to the manufacturer's protocol with slight modifications. Thereafter, probiotic enriched rotifers (n = 3) and control rotifers (n = 3) were examined for bacterial community diversity and composition using Illumina MiSeq of 16S rRNA gene reads amplified from TC-DNA samples. Sequencing was performed at MR DNA (Shallowater, TX, USA) following the manufacturer's guidelines. Sequence data were also processed using MR DNA analysis pipeline.

Statistical analysis: All data regarding larval length, dry weight and survival were submitted to statistical analysis (one-way ANOVA) using "IBM SPSS Statistics v21.0" software (Armonk, NY: IBM Corp.) to assess the existence of significant differences between the two different treatments. The Shapiro-Wilk test was used to assess the normality of the data, and the Leven's test to examine their homogeneity. When the results showed significance ($P < 0.05$), the means between treatments were compared using Tukey's *post hoc* test. A Student's *t*-test was applied to results of survival at the end of the trial.

Results

Taxonomic identification: Using the RDP Seqmatch and NCBI database, the isolates were assigned to six classes (*Alphaproteobacteria, Gammaproteobacteria, Cytophagia, Bacilli,*

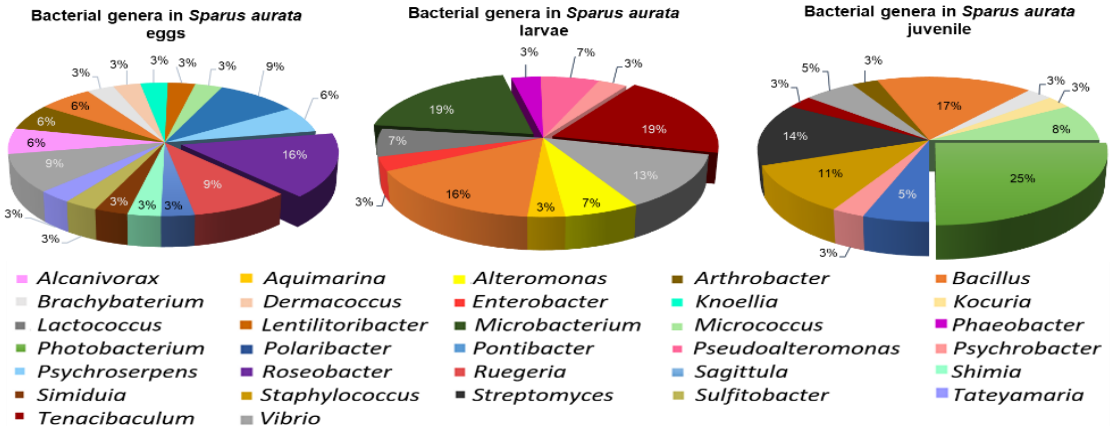

Figure 1. Genus-level composition and 16S rRNA gene-based richness of bacteria isolated from *Sparus aurata* eggs, larvae and juveniles.

Actinobacteria, and *Flavobacteria*) encompassing 32 genera of bacteria (**Figure 1**). While *Proteobacteria* was the dominant phylum across all developmental stages, *Firmicutes* increased and *Bacteroidetes* decreased in abundance in the juvenile stage. The most represented bacterial genera in the egg stage were *Roseobacter* (16%), *Ruegeria* (9%), *Vibrio* (9%), and *Polaribacter* (9%), while in the larval stage the genera *Microbacterium* (19%), *Tenacibaculum* (19%), *Bacillus* (16%), and *Vibrio* (13%), were most dominant. Finally, highest proportions of *Photobacterium* (25%), *Bacillus* (17%), and *Streptomyces* (14%) were found in juvenile guts (**Figure 1**).

Selection of isolates with potential probiotic properties: Based on literature reports, 52 isolates identified in this work were here classified as "potential pathogens" (pathogenicity towards animals or plants, n = 19), "potential probiotics" (already used as probiotics with no evidence of pathogenicity towards animals and plants, n = 5), and "unknown" (all taxa displaying antibacterial compound production, antibacterial activity or host growth enhancement (*e.g.,* plant growth promoters), but for which no information regarding pathogenicity or probiotic activity could be found, n = 28). Only the non-pathogenic and non-redundant isolates belonging to the taxa/species classified as "potential probiotics" and "unknown" were selected for characterization (a total of 45). However, 10 isolates grew poorly in MB medium and could not be fully characterized.

Extracellular enzymes production: Most of the isolates (71%) exhibited at least one hydrolytic activity (**Table 1**). However, only eight isolates (*B. halmapalus* E9, *A. simiduii* L4, *A. gracilis* L21, *A. muelleri* L1, *A. agilis* E13, *B. hwajinpoensis* LB3, *B. oceanisediminis* J2-10 and JB2) had the five hydrolytic activities assessed. In contrast, 10 strains (*S. marina* E8, *R. scottomollicae* E7, *S. porphyrae* E5, *P. inhibens* L23, *P. porphyrae* E23, *S. haliotis* E3, *S. haliotis* E4, *P. marinivivus* E16 and E27, and *T. pelophila* E22) did not show any hydrolytic activity. Relevant lipase activity was observed for isolates *Roseobacter denitrificans* E1 (Enzymatic Activity Index; EAI = 4.94). Several isolates, namely *Bacillus safensis/pumilus* J2-4, *Bacillus plakortidis* JB3, *Aquimarina muelleri* L1, *Bacillus hwajinpoensis* L19 and *Bacillus halmapalus* E9 showed high enzymatic activity index values for protease activity (between 3.5 and 4.0). *Bacillus safensis/pumilus* J2-4 presented the highest protease activity with an EAI of 4.04. Regarding cellulose and starch degradation, *Aquimarina muelleri* L1 presented the highest cellulose and

Table 1. Production of extracellular hydrolytic enzymes (enzymatic activity index). The average for each enzymatic assay (at least biological triplicates) is present in the table. Here are presented only the isolates that revealed at least one hydrolytic activity.

Closest RDP type-strain	ID	Hydrolytic activities (Average ± SD*)				
		Lipases	Cellulases	Proteases	Amylases	Chitinases
Bacillus safensis; B. pumilus	J2-4	2.62 ± 0.24	nd	4.04 ± 0.54	nd	nd
Bacillus halmapalus	E9	2.43 ± 0.43	2.07 ± 0.07	3.57 ± 0.14	1.83 ± 0.17	2.61 ± 0.48
Bacillus plakortidis	JB3	nd	nd	3.79 ± 0.04	nd	nd
Brachybacterium para/conglomeratum	EB1	2.09 ± 0.09	nd	nd	nd	3.38 ± 0.63
Brachybacterium rhamnosum	J9	nd	4.00 ± 0.00	1.69 ± 0.19	2.32 ± 0.18	4.67 ± 0.83
Micrococcus yunnanensis	J8	2.01 ± 0.09	nd	nd	nd	2.85 ± 0.35
Roseobacter denitrificans	E1	4.94 ± 0.96	nd	nd	nd	nd
Alteromonas simiduii	L4	3.44 ± 0.31	3.60 ± 0.15	2.48 ± 0.23	1.46 ± 0.24	5.06 ± 0.30
Alteromonas gracilis	L21	3.02 ± 0.32	4.34 ± 0.06	1.56 ± 0.12	1.90 ± 0.24	3.00 ± 0.49
Psychrobacter nivimaris	LT1	2.79 ± 0.34	nd	nd	nd	nd
Psychroserpens mesophilus	E2	2.39 ± 0.11	nd	1.66 ± 0.22	nd	nd
Aquimarina muelleri	L1	4.11 ± 0.59	5.59 ± 0.73	3.70 ± 0.30	3.76 ± 0.36	4.91 ± 0.80
Pontibacter saemangeumensis	J4	-	2.50 ± 0.50	2.55 ± 0.52	nd	2.73 ± 0.48
Arthrobacter agilis	J16	4.09 ± 0.09	3.48 ± 0.28	2.07 ± 0.07	nd	3.20 ± 0.00
Arthrobacter agilis	E13	3.20 ± 0.53	4.43 ± 1.09	2.96 ± 0.50	3.00 ± 0.20	4.25 ± 0.25
Streptomyces rubrogriseus; S. tendae	J14	2.07 ± 0.38	3.97 ± 0.15	nd	2.58 ± 0.25	3.75 ± 0.25
Streptomyces rubrogriseus; S. tendae	J13	2.67 ± 0.10	4.11 ± 0.74	nd	2.28 ± 0.36	3.29 ± 0.46
Knoellia locipacati	E19	nd	2.79 ± 0.54	nd	nd	nd
Knoellia locipacati	E28	3.16 ± 0.13	4.58 ± 0.89	nd	nd	2.63 ± 0.13
Bacillus hwajinpoensis	LB3	2.33 ± 0.11	1.72 ± 0.06	2.87 ± 0.07	1.24 ± 0.01	2.05 ± 0.11
Bacillus hwajinpoensis	L19	1.96 ± 0.31	1.56 ± 0.06	3.53 ± 0.22	nd	1.93 ± 0.07
Bacillus oceanisediminis	J2-10	1.93 ± 0.07	2.69 ± 0.06	2.35 ± 0.46	1.68 ± 0.14	3.23 ± 0.02
Bacillus oceanisediminis	JB2	2.00 ± 0.00	2.80 ± 0.05	1.97 ± 0.22	1.58 ± 0.22	3.35 ± 0.21
Microbacterium maritypicum	L9	nd	1.96 ± 0.19	nd	nd	2.60 ± 0.40
Microbacterium maritypicum	L24	nd	1.92 ± 0.22	nd	nd	3.00 ± 0.67

"nd", no activity detected; "-", no growth observed; *SD, Standard Deviation.

amylase activity (enzymatic activity index of 5.59 and 3.76, respectively), and the highest chitin-degradation activity (EAI = 5.06) was observed for *Alteromonas simiduii* L4.

Antagonistic activity: In this study, potential probiotic bacteria were also selected based on their antimicrobial activity against pathogens. The antimicrobial activity of the selected isolates (35 strains) was preliminary done by cross-streak assay. Based on the results of this assay, the isolates that showed inhibitory effect on at least one fish pathogenic strain (22 out of the 35 isolates) were selected for further assessment using the soft-agar overlay assay. Based on our results, from the latter (**Table 2**), the isolate *P. inhibens* L23 displayed a wide range of inhibition since it could inhibit the growth of the three pathogens consistently (two Gram-negative bacteria namely *V. parahaemolyticus* (Antagonistic Activity Index; AAI = 1.43) and

Table 2. Antibacterial activity of the selected isolates following the soft-agar overlay method. The average (at least biological duplicates) and the standard deviation (SD) for each antagonistic test is present in the table.

RDP type-strain (Isolates)	Isolate ID	AAI (Average ± SD)		
		Vibrio parahaemolyticus	***Photobacterium Damselae* subsp. *piscicida***	***Streptococcus iniae***
Phaeobacter inhibens	L23	1.43 ± 0.09	2.40 ± 0.10	1.58 ± 0.09
Bacillus oceanisediminis	J2-10	nd	3.00 ± 0.30	nd

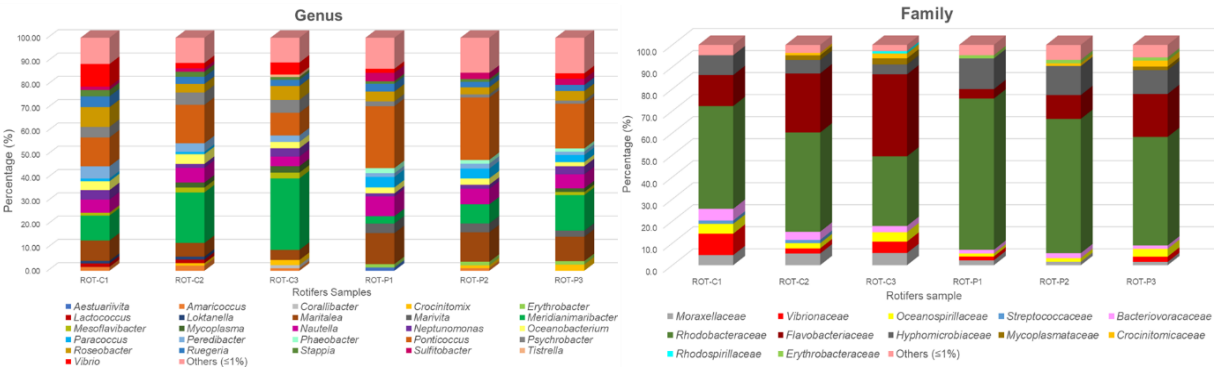

Figure 2. Probiotic enriched rotifers and standard rotifers bacterial community diversity and composition representation (zOTUs), obtained through 16S rRNA gene amplicon sequencing, using Illumina technology. The taxonomic levels such as genus, family, class and phylum, and the taxa with the highest presence (higher than 1%) on the rotifers control samples (ROT-C1, ROT-C2, and ROT-C3) and treated rotifer samples (ROT-P1, ROT-P2, and ROT-P3) are showed. The remaining taxa with a presence lower or equal to 1% was grouped in the category "Others".

Pho. damselae (AAI = 2.40), and one Gram-positive bacterium namely *S. iniae* (AAI = 1.58)). In contrast, the isolate *B. oceanisediminis* J2-10, only displayed antibacterial activity against *Pho. damselae* (AAI = 3.00). The remaining isolates did not show any or consistent results under our experimental conditions and were not considered for further antagonistic assays.

Larval and rotifers trial: Probiotic enriched rotifers (ROT-P) and standard rotifers (ROT-C) were examined for bacterial community diversity and composition through 16S rRNA gene amplicon sequencing, using Illumina technology (**Figure 2**). Despite a small increase in the relative abundance of *A. agilis* and *P. inhibens* in probiotic-enriched rotifers (from an average of 0.01% to 0.04%, and 0.52% to 1.74%, respectively), the addition of these inoculants, although not making them dominant in the system, caused a considerable change in the structure of the microbiomes of the rotifers. For instance, there was an increase in relative abundance of the *Rhodobacteraceae* (with an average increase of almost 20% across the probiotic-treated rotifers, thus dominating the microbiome, **Figure 2-Family chart**). Contrarily, pathogens belonging to the *Vibrio* genus fell in abundance from the ROT-C to the ROT-P, with an average decrease of almost 5%, and genera belonging to the *Flavobacteriaceae* family which is known for harboring opportunistic species [28], also displayed an average decrease of 15% from the ROT-C to the ROT-P samples. Regarding the larvae rearing trial, the total larval length (TL) and dry weight (DW) was similar for both treatments (control *versus* probiotic) not showing statistically significant differences ($P > 0.05$), throughout the trial (**Figure 3**). The survival data analysis also did not show any statistically significant difference between the two treatments ($P > 0.05$), even when calculated from hatching, presenting a survival of 4.55% for control treatment and 5.35% for probiotic treatment, nor, after discarding larvae that died before the mouth opening (**Figure 3**), with a survival of 5.55% for control treatment and 6.53% for probiotic treatment (**Figure 3**).

Discussion

High mortality during fish larviculture is one of the main reasons for economic losses in the aquaculture industry [29]. Several products, such as antibiotics, vaccines, and probiotics, have been introduced in aquaculture management to improve larval survival rates [8, 9, 30]. Among these, probiotic application to control the level of pathogenic bacteria and to promote fish growth has increased in the last decades, since they have been considered a sustainable strategy [31]. However, most of the commercially available probiotics in aquaculture seem to be species-

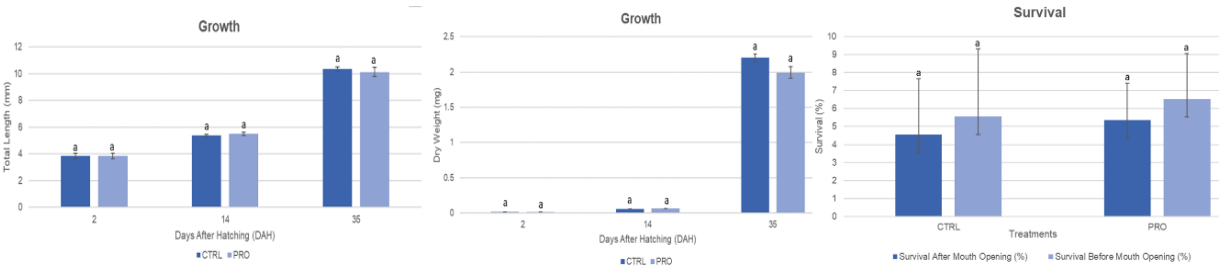

Figure 3. Larval length (mm) and dry weight (mg) biometric data comparison between the control batch (larvae with no potential probiotics; dark blue) and larvae fed with probiotic-treated rotifers (light blue) for samples taken at 2, 14, and 35 DAH is shown. The larval survival from the 1 DHA (before mouth opening) is shown as well as the larval survival from the 3 DHA (after mouth opening). The standard deviation is presented for all data. All statistical analysis were performed using "IBM SPSS Statistics 21.0 software". The data referring to the growth of different treatments were submitted to an unidirectional variance analysis (one-way ANOVA), to evaluate the existence of significant differences between the two different treatments. Statistical significance was assessed with a confidence level of 95%. No statistical difference between the treatment and control tanks was observed, indicated in the figure by the letter "a" on top of each bar.

specific or from non-fish sources and, therefore, colonization, survival rate, and efficacy of these probiotics in the fish gut might be questionable. Thus, the identification of novel probiotics from the fish host is a timely demand in this field of research [32]. In the present study, a total of 97 strains isolated from different *S. aurata* developmental stages (eggs, larvae, and juvenile guts) were analyzed. These strains belonged to four phyla: *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Actinobacteria*. Employing a culture-independent approach, Califano *et al.*, (2017) [33] and Nikouli *et al.*, (2019) [34] reported, in general, similar results to the ones obtained in this study (*Proteobacteria* was the dominant phylum across all development stages and *Firmicutes* increased in abundance in the juvenile stage). However, these studies found 34 (Califano *et al.*, (2017)) and 19 (Nikouli *et al.*, (2019)) bacterial phyla, which is nine times and almost five times more, respectively, than the ones found in the present study (four phyla) [33, 34]. Likewise, when comparing genera among Califano *et al.*, (2017) and the present study, significant changes between both methods were found. For example, Califano *et al.*, (2017) found genera such as *Loktanella, Actinobacillus, Paracoccus,* and other uncultured bacteria in higher abundance than in the present study [33]. In contrast, genera such as *Vibrio, Photobacterium, Roseobacter, Ruegeria, Bacillus, Tenacibaculum, Streptomyces,* and *Microbacterium* were found at a much higher proportion when using a culture-dependent method rather than molecular methods. In fact, most of these genera were virtually not found by molecular techniques, mainly the pathogenic genera such as *Vibrio, Tenacibaculum* and *Photobacterium*.

Based on the data and results obtained during this project, the two isolates with the best activities from each assay (hydrolytic and antagonistic) were chosen to attempt a complementary and balanced action of the putative probiotics. The isolates with better hydrolytic activity in general (high activity in all enzymatic tests) were *A. muelleri* L1 and *A. agilis* E13. However, despite exhibiting better results, isolate L1 was not selected since some species of the *Aquimarina* genus were found to be opportunistic pathogens in lobsters, for example by degrading the chitin shell of these marine animals, through chitinolytic activity [35]. Thus, isolate E13 was chosen instead. The isolate with the best antagonistic activity (antibacterial activity against all three indicator strains) was *P. inhibens* L23. Therefore, this isolate was also chosen for the larval rearing trial. Using three independent replicates for probiotic-treated and control rotifers sample groups, obtained during the larval trial, it was clear that the treated rotifers

presented a significant increase of *Rhodobacteraceae* [36], and a significant decrease of known pathogenic (and opportunistic) species [37], likely due to the combining bioactivities of the two added isolates.

Rotifers are crucial for the fish/larvae health not only by potentially acting as vehicle of bacteria (beneficial or pathogenic), but also as a means for growth and durable life [37]. Manipulation of the microbiomes associated with the live feed provided to the larvae has potential application in the delivery of probiotics onto reared species. Zink *et al*., (2013) [38] demonstrated that not only it is possible to apply probiotics in live feed, but it is also possible to use probiotics as an approach to improve rotifers. In the mentioned study, the authors assessed a commercially available *Bacillus* spp. probiotic blend on population growth dynamics of the rotifer *Brachionus plicatilis*. This addition improved rotifer culture population growth rate, suggesting potential production benefits from its exploitation [38]. Optimum inoculation densities for host-associated probiotics must be carefully determined to avoid overdosing, which could result in lower efficacy while increasing costs. Moreover, the fish, or the fish microbiome, may be too sensitive to the potential probiotics used [32]. A study conducted by Grotkjær *et al.*, (2016) [39] determined that a concentration of 10^6 cfu/mL was sufficient for *P. inhibens* to maintain its cell densities throughout *Artemia* culture trials, independently of the background microbiome [39]. Hence, the bacterial density used in this study (10^6) was very close of the optimal and suggested bacterial density.

At the beginning of this work the hypothesis/question if it was feasible to manipulate the live feed microbiome was proposed. The results obtained here revealed this hypothesis to be true. Moreover, the shift present in the rotifers communities might also clarify if a change in the larvae microbiome might have occurred, that is, an alteration in the larvae microbiome from a detrimental state, with the presence of several pathogenic bacteria, to a more benefic environment with for example *Rhodobacteraceae* standing out and controlling the microbiome, while having a positive effect on larvae overall biometrics and survival (analysis being carried at the moment).

Despite no statistical difference was observed for the biometric and survival data among control and treatment groups, slight survival increases of 0.80% were seen when calculated from hatching and of 0.98% when evaluated after mouth opening (with a significant lower standard deviation), which might have happened due to the positive action of the added isolates. In the present work, the low survival of the treated larvae group might be related with several factors (*e.g.*, lack of potential probiotics intake by the fish (selective ingestion), absence of probiotic activity, or incorrect dosage, dosage frequency, and duration for bacterial colonization and consequent growth). Even so, the decrease of the relative abundance of pathogens in the live feed under probiotic treatment might suggest that pathogen control may be possible using both strains and/or that the larvae may be more resistant to a potential disease outbreak. To fully confirm and answer these questions, an 16S rRNA gene amplicon sequencing of the larval samples taken during the larval trial are being performed at the moment to assess the larvae bacterial community profiles.

Conclusion

The work conducted here allowed the identification of several novel fish-associated bacterial strains possessing bioactivities

presumably related to the fish growth promotion and biocontrol. From these results, it was determined that the two isolates most appropriate for the desirable outcome (increase and enhance larval survival and biometrics, respectively) were *Phaeobacter inhibens* L23 and *Arthrobacter agilis* E13. Together, these isolates were expected to complement each other in the larval trial, through the application of their bioactivities (antagonism and hydrolytic enzymes production by the *P. inhibens* and *A. agilis*, respectively) on the live feed (rotifers), eggs and larvae.

Rotifers are considered one of the most suitable prey for a first feeding and one of the most important vectors of bacteria to the larvae, whether beneficial or harmful [37]. As a result, in a future research project or in a future continuity of this project the study should, firstly, evaluate the best vehicle (*e.g*., live feed or water) to transport the isolate to the eggs/larvae. Here, it was demonstrated that the bacterial strains used as putative probiotics positively influenced and altered the structure of the rotifers microbiome, despite not being dominant in the microbiome. Therefore, further studies must focus on the isolates ability to colonize and thrive on the live feed microbiome, for example, through the use of different inoculum densities, and consequent effects of the isolates on the feed growth, mobility, survival and overall microbiome. Only then, idealistically, the research should go through an *in vivo* larviculture trial (from the eggs to the larvae) to assess the larvae biometric and survival data, and the gut bacterial content.

This is the first report (as far as we know) to isolate and characterize cultivable bacteria from *S. aurata* and is also the first study to apply either of the isolates (*P. inhibens* and *A. agilis*) in this particular fish larval trials.

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References

1. Mittal, I. and R.K. Gupta, Natural Resources Depletion and Economic Growth in Present Era. SOCH-Mastnath Journal of Science & Technology (BMU, Rohtak), 2015. **10**(3).
2. Stentiford, G.D., *et al*., Disease will limit future food supply from the global crustacean fishery and aquaculture sectors. J Invertebr Pathol, 2012. **110**(2): p. 141-57.
3. Toranzo, A.E., B. Magariños, and J.L. Romalde, A review of the main bacterial fish diseases in mariculture systems. Aquaculture, 2005. **246**(1-4): p. 37-61.
4. Benetti, D.D., *et al*., Site selection criteria for open ocean aquaculture. Marine Technology Society Journal, 2010. **44**(3): p. 22-35.
5. Chua, T.-E., Coastal aquaculture development and the environment: the role of coastal area management. Marine Pollution Bulletin, 1992. **25**(1-4): p. 98-103.
6. De Silva, S.S., *et al*., An evaluation of the role and impacts of alien finfish in Asian inland aquaculture. Aquaculture Research, 2006. **37**(1): p. 1-17.
7. Cochrane, K., *et al*., Climate change implications for fisheries and aquaculture. FAO Fisheries and aquaculture technical paper, 2009. **530**: p. 1-218.
8. Li, X., *et al*., Aquaculture industry in China: current state, challenges, and outlook. Reviews in Fisheries Science, 2011. **19**(3): p. 187-200.
9. Cole, D.W., *et al*., Aquaculture: Environmental, toxicological, and health issues. International journal of hygiene and environmental health, 2009. **212**(4): p. 369-377.

10. Scarano, C., *et al*., Antibiotic resistance of *Vibrio* species isolated from *Sparus aurata* reared in Italian mariculture. New Microbiol, 2014. **37**(3): p. 329-337.
11. Comission, E. European Comission Gilthead seabream. 2012 20 may, 2021]; Available from: http://ec.europa.eu/fisheries/marine_species/farmed_fish_and_shellfish/seabream/index_en.htm.
12. Picone, G., *et al*., Metabolomics as a powerful tool for molecular quality assessment of the fish *Sparus aurata*. Nutrients, 2011. **3**(2): p. 212-27.
13. Muniesa, A., *et al*., Mapping the knowledge of the main diseases affecting sea bass and sea bream in Mediterranean. Transboundary and emerging diseases, 2020. **67**(3): p. 1089-1100.
14. Ghittino, C., *et al*., Emerging pathologies in aquaculture: effects on production and food safety. Veterinary Research Communications, 2003. **27**(1): p. 471-479.
15. Marchesi, J.R. and J. Ravel, The vocabulary of microbiome research: a proposal. Microbiome, 2015. **3**: p. 1-3.
16. Swanson, K.S., *et al*., The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of synbiotics. Nature Reviews Gastroenterology & Hepatology, 2020. **17**(11): p. 687-701.
17. Sahu, M.K., *et al*., Probiotics in aquaculture: importance and future perspectives. Indian J Microbiol, 2008. **48**(3): p. 299-308.
18. Khochamit, N., *et al*., Antibacterial activity and genotypic–phenotypic characteristics of bacteriocin-producing *Bacillus subtilis* KKU213: Potential as a probiotic strain. Microbiological Research, 2015. **170**: p. 36-50.
19. Kumar, D., *et al*., Screening, isolation and production of lipase/esterase producing *Bacillus* sp. strain DVL2 and its potential evaluation in esterification and resolution reactions. Archives of Applied Science Research, 2012. **4**(4): p. 1763-1770.
20. Akpan, I., M. Bankole, and A. Adesemowo, A rapid plate culture method for screening of amylase producing micro-organisms. Biotechnology techniques, 1999. **13**(6): p. 411-413.
21. Sugita, H., J. Kawasaki, and Y. Deguchi, Production of amylase by the intestinal microflora in cultured freshwater fish. Letters in Applied Microbiology, 1997. **24**(2): p. 105-108.
22. Bhaskar, N., *et al*., Partial purification and characterization of protease of *Bacillus proteolyticus* CFR3001 isolated from fish processing waste and its antibacterial activities. Bioresource Technology, 2007. **98**(14): p. 2758-2764.
23. Vermelho, A.B., *et al*., Detection of extracellular proteases from microorganisms on agar plates. Memorias do Instituto Oswaldo Cruz, 1996. **91**(6): p. 755-760.
24. Tanaka, D., *et al*., Isolation of cellulase-producing *Microbulbifer* sp. from marine teleost blackfish (*Girella melanichthys*) intestine and the enzyme characterization. The Journal of General and Applied Microbiology, 2020: p. 1-23.
25. Hamid, R., *et al*., Chitinases: an update. Journal of pharmacy & bioallied sciences, 2013. **5**(1): p. 21.
26. Abe, C.A.L., *et al*., Fungi isolated from maize (*Zea mays L*.) grains and production of associated enzyme activities. International journal of molecular sciences, 2015. **16**(7): p. 15328-15346.
27. Castanho, S.I.C., The potential of *Asparagopsis armata* to control the bacterial load associated to live feed to improve Seabream (*Sparus aurata*) larvae performance, in Mestrado em Aquacultura e Pescas. 2014, Universidade do Algarve (Faculdade de Ciências e Tecnologia). p. 66.
28. McDevitt-Irwin, J.M., *et al*., Responses of coral-associated bacterial communities to local and global stressors. Frontiers in Marine Science, 2017. **4**: p. 262.
29. Castanho, S., *et al*., The effect of live feeds bathed with the red seaweed *Asparagopsis armata* on the survival, growth and physiology status of *Sparus aurata* larvae. Fish Physiol Biochem, 2017. **43**(4): p. 1043-1054.
30. Sommerset, I., *et al*., Vaccines for fish in aquaculture. Expert review of vaccines, 2005. **4**(1): p. 89-101.
31. Irianto, A. and B. Austin, Probiotics in aquaculture. Journal of fish diseases, 2002. **25**(11): p. 633-642.
32. Van Doan, H., *et al*., Host-associated probiotics: a key factor in sustainable aquaculture. Reviews in fisheries science & aquaculture, 2020. **28**(1): p. 16-42.
33. Califano, G., *et al*., Molecular Taxonomic Profiling of Bacterial Communities in a Gilthead Seabream (*Sparus aurata*) Hatchery. Frontiers in Microbiology, 2017. **8**(204): p. 1-16.
34. Nikouli, E., *et al*., Host-Associated Bacterial Succession during the Early Embryonic Stages and First Feeding in Farmed Gilthead Sea Bream (*Sparus aurata*). Genes, 2019. **10**(7): p. 1-15.
35. Midorikawa, Y., *et al*., Characterization of *Aquimarina hainanensis* isolated from diseased mud crab *Scylla serrata* larvae in a hatchery. Journal of fish diseases, 2020. **43**(5): p. 541-549.
36. Borges, N., *et al*., Bacteriome Structure, Function, and Probiotics in Fish Larviculture: The Good, the Bad, and the Gaps. Annual Review of Animal Biosciences, 2020. **9**: p. 15.1-15.30.
37. Chen, J.Y., *et al*., Recent advances of marine ornamental fish larviculture: broodstock reproduction, live prey and feeding regimes, and comparison between demersal and pelagic spawners. Reviews in Aquaculture, 2020. **12**(3): p. 1518-1541.
38. Zink, I.C., P.A. Douillet, and D.D. Benetti, Improvement of rotifer *Brachionus plicatilis* population growth dynamics with inclusion of *Bacillus* spp. probiotics. Aquaculture Research, 2013. **44**(2): p. 200-211.
39. Grotkjær, T., *et al*., *Phaeobacter inhibens* as probiotic bacteria in non-axenic *Artemia* and algae cultures. Aquaculture, 2016. **462**: p. 64-69.