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In Vitro Selection of Bacteria and Isolation of Probionts from Farmed *Sparus aurata* with Potential for Use as Probiotics

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Abstract

In the last years aquaculture has contributed significantly to reduce the hunger worldwide. One of the major treats in the development of a massive production is linked to the outbreak of diseases and the abuse of antibiotics, that is to avoid because of the acquisition of antibiotic resistance. For these reasons recently probiotics are used as alternative measures to control the fish diseases. Fish possess specific intestinal microbiota consisting of aerobic, facultative anaerobic and obligate anaerobic bacteria so we make this study in order to find some probiotic candidates that have an antagonistic action against fish pathogens. Adults of *Sparus aurata* farmed in intensive plant were sacrificed and 40 bacterial strains were isolated from GI tract. All the strains were tested against three fish pathogens: *Vibrio anguillarum*, *Photobacterium damselae subsp. piscicida* and *Pseudomonas anguilliseptica.* Results showed that only 3 candidates respectively called (SA7, SA10, and SA20) showed an inhibitory activity against the selected fish pathogens bacteria. The candidates probionts were identified by the 16S rRNA gene sequenced-based. The three candidates inhibited the Gram negative fish pathogens after 24 -48 h of incubation at 24°C and for these reasons could be used as probiotics to added into the food to enhance the immune defence of fish.

Keywords

Antimicrobial Effects, Fish Pathogens, Microbial Gut, Probiotics, Seabream

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1. Introduction

The aquaculture, in the last years, contributed significantly to reduce the hunger and malnutrition worldwide, becoming an economically important industry (Subasinghe et al., 2009). FAO estimates to feed the world in 2050 must increase by over 60%. The production is maximized through intensification with addition of commercial diets, growth promoters, antibiotics, and several other additives; all these practices create stressful conditions that cause problems related to diseases and deterioration of environmental conditions often occur and result in serious economic losses (Panigrahi and Azad, 2007; Mancuso, 2013a). The prevention and the control of diseases have led during recent decades to a substantial increase in the use of veterinary medicines. The massive use of antibiotics for the control of diseases has been questioned by acquisition of antibiotic resistance in disease causing agents and the need of alternative measures to control these diseases is of prime importance (Mancuso, 2013b). The interest in probiotics as an environmentally friendly alternative is increasing and its application is both

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empirical and scientific (Soccol et al., 2010). In recent years, probiotics have a center stage and are used as alternative measures to control the fish diseases; in fact they inhibit pathogenic microorganisms and have been used therapeutically to treat a variety of gastrointestinal and even systemic disorders (Maricchiolo et al., 2015, Maricchiolo et al., 2014). Probiotics transiently colonize the bowel and except when used to treat an acute disorder, must be regularly consumed to maintain benefit. Use of microbial probiotics to promote health maintenance and disease prevention and control is now widely accepted as the new ecofriendly alternative measures for sustainable aquaculture (Ram and Parvati, 2012; Mancuso, 2013b). Fish possess specific intestinal micro-biota consisting of aerobic, facultative anaerobic and obligate anaerobic bacteria. These bacteria are responsible for enteric bacterial antagonism and colonization resistance, since they are associated closely with the intestinal epithelium, and form a barrier, serving as the first defence to limit direct attachment or interaction of fish pathogenic bacteria to the gut mucosa. Numerous surveys of the bacterial flora in the GI tract of fish have been made during the last twenty years. Many reports have demonstrated that Gram-negative, facultative anaerobic bacteria such as *Acinetobacter, Alteromonas, Aeromonas, Bacteroides, Cytophaga, Flavobacterium, Micrococcus, Moraxella, Pseudomonas, Proteobacterium* and *Vibrio* spp. constitute the predominant endogenous microbiota of a variety of species of marine fish (Cahill, 1990; Zhou et al., 2009). Various species of lactic acid bacteria (LAB) (*Lactobacillus, Lactococcus, Streptococcus, Leuconostoc*, and *Carnobacterium* spp.) have been also demonstrated to comprise part of this microbiota (Ringø and Gatesoupe, 1998; Vendrell et al., 2006; Balcázar et al., 2008). The GI microbiota in fish is variable based on: nutrition, intestinal microenvironment, age, geographical location, environmental factors, stress (Verschuere et al., 2000, Kesarcodi-Watson et al., 2008, Mancuso, 2013). The intestinal microbiota has important and specific metabolic, trophic, and protective functions (Guarner and Malagelada, 2003). The normal gut microbiota confers many benefits to the intestinal physiology of the host. Some of these benefits include the metabolism of nutrients, contribution of the colonization resistance, antagonistic activity against pathogens, immunomodulation and etc. (Denev, 1996, Decamp and Moriarthy, 2007). The intestinal microbiota has a profound impact on the anatomical, physiological and immunological development of the host. Thus, establishing a healthy microbiota plays an important role in the generation of immuno-physiologic regulation by providing crucial signals for the development and maintenance of the immune system (Salminen et al., 2005). Understanding how the fish immune system generally responds to gut microbiota may be an important basis for targeting manipulation of the microbial composition. This might be of special interest to design adequate strategies for fish disease prevention and treatment (Gomez and Balcázar, 2008). The intestinal microbiota possesses antagonistic activity against many fish pathogens and participates in infection-protective reactions (Gutowska et al., 2004). Yoshimizu and Ezura (1999) reported that fish intestinal bacteria such as *Aeromonas* and *Vibrio* spp. produced antiviral substances.

The aim of our study was to find some probiotic candidates that have an antagonistic action against fish pathogens.

2. Materials and Methods

2.1. Isolation of Candidate Probionts

10 healthy adults of *Sparus aurata* farmed in intensive plant were dissected, previous euthanasia with a lethal dose of MS222 (0,5g/L) (Sigma-Aldrich). The fish were kept in starvation for 48 h prior to sacrifice in order to clear their gastrointestinal tract (GI). the ventral surface was sterilized using 70% ethanol and dissected aseptically to remove the intestine. The gut was collected and samples were divided into PI (proximal intestine) and DI (distal intestine) and processed for isolation of autochthonous microorganisms and homogenated in 10 ml of sterile saline solution. Serial dilutions were made of each sample and plated in: Marine Agar (Microbial diagnostic), TSA (added with 1.5% NaCl final concentration) (Oxoid), MacConkey agar (Oxoid) and TCBS agar (Oxoid), following the Vine et al. (2004) protocol. All plates were incubated for 25°C from 24-48h up to 10 days after which colony-forming units (CFU) were counted. Counts between 30 and 300 CFU were used for analysis.

2.2. Pathogen Collection and Culture Conditions

A study of the bacterial growth inhibition to test for the production of antimicrobial metabolites by the isolates was performed using three fish pathogens: *Vibrio anguillarum*, *Photobacterium damselae subsp. piscicida* and *Pseudomonas anguilliseptica* (kindly furnished by Dr Amedeo Manfrin IZS of Venice).

Fish pathogenic strains were inoculated (10^8 cells 100 μL^{-1}) by pour plating and separately grown on TSA media (added with 1.5% final con concentration of NaCl - Oxoid) plates. Agar wells were cut into the agar and filled with 0.1 ml of the marine broth putative probiotic isolates and were incubated for 24-48 h at 24°C. Appearance of zones of inhibition (halo, diameter in mm) around the wells were recorded and presented accordingly. The presence of antimicrobial metabolites produced by the isolates inhibited the growth of the pathogen producing a zone of inhibition around the well.

After the incubation time a clear zone of inhibition (halo) around growth of the selected gut bacteria indicated antibacterial activity and the halo zone (diameter in mm) around the colony was presented as scores as follows; 0 (0–5 mm), 1 (low, 6–10 mm), 2 (moderate, 11–20 mm), 3 (high, 21–25 mm) and 4 (very high, ≥26 mm) (Mukherjee and Ghosh, 2014).

2.3. DNA Extraction, 16S rRNA Gene Sequencing and Phylogenetic Analysis

The candidates probionts were identified by the 16S rRNA gene sequenced-based.

Analysis of the 16S rRNA gene was performed for the taxonomic characterization of the isolated strains. Total DNA was extracted from the bacterial strains using Qiagen RNA/DNA Mini Kit (Qiagen, Milan, Italy). The extraction was carried out according to the manufacturer's instructions. DNA samples was examined by agarose gel electrophoresis and concentrations was determined using the the NanoDrop® ND-1000 spectrophotometer (Celbio). DNA was used as template for further analysis. The bacterial 16S rRNA loci were amplified using the domain-specific forward primer Bac27_F (5′-AGAGTTTGATCCTGGCTCAG-3′) and the universal reverse primer Uni_1492R (5′-TACGYTACCTTGTTACGACTT-3′) (Lane 1991) . The amplification reaction was performed in a total volume of 50 µl containing 1× solution Q (Qiagen, Hilden, Germany), 1× Qiagen reaction buffer, 1 µM of each forward and reverse primer, 10 µM dNTPs (Gibco, Invitrogen Co., Carlsbad, CA), and 2 U of Qiagen Taq polymerase (Qiagen). Amplification for 35 cycles was performed in a GeneAmp 5700 thermocycler (PE Applied Biosystems, Foster City, CA, USA). The temperature profile for PCR was 95 °C for 5 min (1 cycle); 94 °C for 1 min and 72 °C for 2 min (35 cycles); and 72 °C for 10 min after the final cycle . PCR product was purified and sequenced using Macrogen Service (Macrogen, Korea) (Genovese et al., 2014). The analysis of the sequences (1000 bp of average length) was performed as following described.

The similarity rank from the Ribosomal Database Project RDP) (Maidak et al., 1997) and FASTA Nucleotide Database Queries were used to estimate the degree of similarity to other 16S rRNA gene sequences. Phylogenetic analysis of the sequences was performed as previously described by Yakimov et al. (2006).

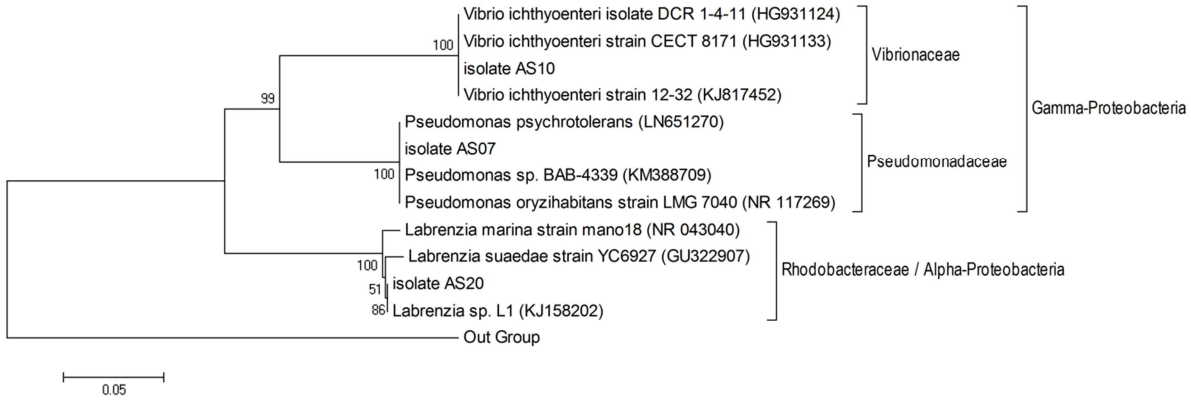

Figure 1. Phylogenetic tree based on 16S rRNA gene sequences for bacterial strains (isolates AS-07, -10 and -20). Percentages of 100 bootstrap resampling that supported the branching orders in each analysis are shown above or near the relevant nodes. The tree was rooted and outgrouped by using the 16S rRNA sequences of *Methanococcus jannaschii* (M59126). Evolutionary distance is indicated by vertical lines; each scale bar length corresponds to 0.05 fixed point mutations per sequence position.

3. Results

Bacterial cell concentrations based on CFU isolated from the gut in marine agar was: $7*10^6$CFU/ml. Mc Conkey: 0, TCBS: $3*10^5$CFU/ml (pictures not shown).

In total were isolated 40 bacteria from GI tract (respectively 20 for PI and 20 for DI) and were tested against the fish pathogens bacteria. Only 3 candidates respectively called (SA7, SA10, and SA20) showed an inhibitory activity against the selected fish pathogens bacteria.

Candidate probiotic SA 10 showed the greatest antagonistic activity against *Vibrio anguillarum* with an inhibition halo of 21 mm. The lowest halo was from SA20 that showed on 15 mm.

The halos against *Photobacterium damselae subspecie piscicida* were the lowest in absolute with 10 to 12 mm halos and against *Pseudomonas anguilliseptica* all candidates showed the same halo 13 mm (Table 1).

16S rRNA gene sequencing analysis

The molecular identification of isolates was performed amplifying and sequencing the 16S rRNA gene and comparing the sequences to the database of known 16S

rRNA sequences. The results are shown in Figure 1. Two isolates (AS7 and AS10) belong to Gamma-Proteobacteria class (Pseudomonadaceae and Vibrionaceae family, respectively) and a isolate AS20 belong to group of Alpha-Proteobacteria (Rhodobacteraceae)

Table 1. Antibacterial activity of probiotic candidates with halos expressed in mm.

Candidates	*Vibrio anguillarum*	*Photobacterium damselae subsp. piscida*	*Pseudomonas anguilliseptica*
SA 7	20 mm	10 mm	13 mm
SA10	21 mm	12 mm	13 mm
SA 20	15 mm	12 mm	13 mm

In particular, the isolate AS7 related to *Pseudomonas psychrotolerans* (LN651270, 99% ID), the strain AS10 to *Vibrio ichthyoenteri* strain 12-32 (KJ817452, 99% ID) and AS20 belonged to *Labrenzia* sp. L1 (KJ158202, 99% ID). The sequences of the bacteria in study were submitted to the genetic sequence database at the National Center for Biotechnical Information (NCBI).

4. Discussion

Bacteria are the most common among the pathogens in cultured fish that cause mass mortality in aquaculture both marine and freshwater (Mancuso, 2014; Mancuso, 2013 (a,b,c); Mancuso, 2012, Mancuso, et al. 2013; Mancuso, et al. 2005; Zaccone, et al.2004) for this reason the antibiotics are used to treat these diseases. The use of these substances can cause: environmental problems (Martinez, 2012), the development of drug-resistant bacteria (Nomoto, 2005) and the accumulation of residues in fish tissues (Chevassus and Dorson, 1990). For these reason it is necessary to develop alternative ways to combat the diseases (Martinez-Cruz et al., 2012). In recent years, the research of pro- and prebiotics in fish nutrition is increasing with the demand for consumer and environment-friendly aquaculture (Denev et al., 2009). The production of antimicrobial substances by some bacteria seemed to play an important role in antagonizing other bacteria in aquatic ecosystems (Dopazo et al., 1988).

In this study the gut microbiota of seabream was screened for the research of putative probiotic bacteria. Based on partial 16S rRNA gene sequencing, the isolates were defined into 3 bacterial groups, respectively AS7 belongs to Pseudomonaceae, AS10 to Vibrionaceae and AS20 to Rodobacteriaceae, moreover AS7 and AS10 belongs to Gamma Proteobacteria, while AS20 to Alpha Proteobacteria. The three candidates inhibited the Gram negative fish pathogens after 24 -48 h of incubation at 24°C.

Considering antagonism towards pathogens and verification of other probiotic properties, 3 bacterial isolates were characterized as putative probiotics.

The isolate AS7 was identified as *Pseudomonas psychrotolerans* is normally present into the gut bacterial flora as reported by (Floris et al., 2013).

The isolate AS10 was identified as *Vibrio ichthyoenteri* as previously showed from healthy fish (Floris et al 2013) and finally the isolate AS20 was identified as *Labrenzia sp.* present normally in sea waters (Biebl et al., 2007).

Previous studies reported that bacilli isolated from intestines of Japanese costal fish (Sugita *et* al., 1998) and an Indian Major Carp, Labeo rohita (Giri et al., 2012, 2013) produced antimicrobial substances produced. And also Pseudomonas species and Vibrio sp. have some antagonistic activities against fish pathogens, respectively (Das et al., 2006) and (Vijayan et al., 2006).

The present study, to our knowledge, is the first carried out on adults of sea bream the selected isolates from the gut of seabream were antagonistic to 3 fish pathogens that included *Vibrio anguillarum*, *Photobacterium damselae subsp. piscicida* and *Pseudomonas anguilliseptica.*

In this study we found that 3 bacteria isolated from microbial gut of *Sparus aurata* could be used as probiotic candidates to added into the food to enhance the immune defence of fish. Finally this could be a starting point for further studies to verify the effectiveness and protection against bacterial diseases during an experimental challenge.

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