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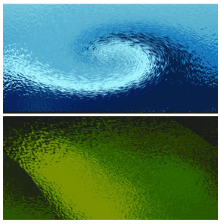

RESEARCH ARTICLE

Microbial ecology of intestinal tract of gilthead sea bream (*Sparus aurata* Linnaeus, 1758) from two coastal lagoons of Sardinia (Italy)

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Abstract

1 - The bacterial flora of the digestive tract of aquatic organisms reflects various factors, such as the aqueous environment (temperature, salinity, etc.), seasonal variation, diet, fish species and anatomy of gastrointestinal section.

2 - In the present work, culturable bacteria isolated from intestinal samples of gilthead sea bream caught in two coastal lagoons of Sardinia, were quantified and identified in order to detect the effect of different habitats on the microbial ecology of fish gut.

3 - A total of 120 bacterial colonies coming from intestinal tracts of *Sparus aurata* specimens captured in the Tortoli (Eastern Sardinia: Lat 39°56' 854''N, Long 9°41'160''E) and Porto Pino (Southern Sardinia: Lat 39°02' 54''N, Long 08°32'54''E) lagoons during the winter season, were identified by means of amplified ribosomal DNA restriction analysis and 16S rRNA gene sequencing.

4 - The results showed no significant differences in the bacterial loads, while a diverse composition of microbial gut flora was detected between the two groups of gilthead sea bream. Indeed, intestinal microbiota from the Tortoli lagoon showed high genetic variation with a total of 13 different taxonomic bacterial groups identified as *Pseudomonas* spp. (33.3%), *Sphingomonas paucimobilis* (10.5%), *Proteus* spp. (8.8%), *Chryseobacterium* sp. B-G-R2A3 (5.3%), Arctic soil bacterium A1T3 (5.3%), *Sphingobacterium* spp. (5.3%), *Psychrobacter* spp. (3.5%), *Psychrobacter maritimus* (3.5%), *Leucobacter* spp. (3.5%), *Yersinia bercovieri* (3.5%), *Aeromonas* spp. (3.5%), *Aeromonas molluscorum* (1.7%), and *Erwinia persicina* (1.7%). On the other hand, 16S rRNA gene analyses of bacterial flora performed on the Porto Pino gut samples, revealed a lower variability when compared with those from Tortoli, since only 3 different taxonomic groups were distinguished and ascribed to *Pseudomonas* spp. (90%), *Janthinobacterium* spp. (8%) and *Psychrobacter maritimus* (2%).

5 - Our findings indicate that the aqueous habitat highly selects fish microbial gut flora which represents a peculiar ecosystem and a possible biomarker of environmental origin.

Keywords: gut microflora, *Sparus aurata*, 16S rRNA gene, Sardinian lagoons.

Introduction

A large community of bacteria inhabits the gastrointestinal tract of all animals and forms a closely integrated ecological unit with the host (Skrodenyte-Arbaciauskiene *et al.*, 2008). These microbiota in fish constitute an important component of the digestive duct system which plays an important role in the host's health and quality (Carnevali *et al.*, 2006; Abelli *et al.*, 2009) and takes part in various digestive processes (Bairagi *et al.*, 2002; Fidopiastis *et al.*, 2006) like the synthesis of bioactive compounds, such as eicosapentaenoic acid, and short-chain fatty acids (SCFAs) (Kihara and Sakata 2001, 2002; Mahious *et al.*, 2006).

Much attention has been paid in scientific literature to the composition of the intestinal microbiocenosis of various fish species under the effect of abiotic and biotic factors, since it provides information about the way of life of the host and the quality of the environment (Al-Harbi and Uddin, 2004; Izvekova *et al.*, 2007). Most microbiological studies have referred to aquatic specimens from Northern Europe, in particular the rainbow trout (Kim *et al.*, 2007), Atlantic cod (Ringø *et al.*, 2006), Atlantic salmon (Ringø *et al.*, 2008) and gilthead sea bream from aquaculture plants fed different diets (Dimitroglou *et al.*, 2010). Studies on the microbial ecology of the intestinal tract of fish specimens inhabiting Mediterranean natural coastal lagoons are important in the light of the fact that these aquatic environments represent about 15,000 hectares of the island of Sardinia and among them 10,000 are considered areas of ecological interest with respect to their protection and conservation both at regional and international level according to the jurisdiction of the European Water Framework Directive (WDF; 2000/60/EC).

There are few reports regarding the isolation and identification of cultivable aerobic or aerotolerant intestinal bacteria from species such as *Sparus aurata* inhabiting Mediterranean aquatic areas. Previous studies regarding the quali-quantitative characterization of the intestinal microflora of *Sparus aurata* reared in Sardinian aquaculture systems have highlighted the fact that it is composed of a complex and variable bacterial community (Floris *et al.*, 2011a). The aim of the work reported in this paper was the quantification and identification of the culturable microbial communities associated with the gut of gilthead sea bream captured in two ecologically different Sardinian coastal lagoons, in order to evaluate the effect of these diverse habitats on the microbial ecology of fish gut and to assess if these microbiota can be considered biomarkers of environmental origin.

Materials and Methods

Study site

The Tortoli lagoon is located on the eastern coast of the region of Sardinia (Lat 39°56' 854''N, Long 9°41'160''E) (Fig.1). It covers an area of 250 ha, possesses two entries from the sea and a depth of 1 to 4 m. The waters are classified as eutrophic and the mean winter hydrological parameters are as follows: temperature 14°C, salinity 35‰, dissolved oxygen 96%, pH 8.14. The Porto Pino salty ponds are classified as oligotrophic lagoons and are situated in southern Sardinia (Lat 39°02' 54''N, Long 08°32'54''E) (Fig.1). They comprise a 440 ha area complex of 3 shallow basins (89 ha La Spiaggia, 162 Is Brebeis, and 189 Maestrale) which are connected to each other with only La Spiaggia communicating directly with the sea (Rossi and Cannas, 1982). The mean winter hydrological parameters are as follows: temperature 18°C, salinity 37‰, dissolved oxygen 82%, pH 7.8.

Microbiological analyses

Sampling and processing

A total of 10 commercial size gilthead

Figure 1. Geographic locations of Sardinian coastal lagoons representing the sampling sites (expanded views on the lower level) situated in the Central and Western Italian Mediterranean Sea.

sea bream (five from each lagoon) (mean weight 356.4 ± 37 g for the Tortoli fish and 313.5 ± 30 g for the Porto Pino fish) were collected in two winter seasons, in 2009 and 2011. Subsequent to capture, the gilthead seabream were stored immediately in ice and transported inside a refrigerated bag to Bonassai laboratory within 6-8 h. Following a series of preliminary studies to optimise the methodology, a sampling and processing protocol was defined. On arrival at the laboratory, the fish were weighed, measured and after sterilization of the belly by flame, the peritoneal cavity was aseptically opened with a sterile blade. The intestine between the pyloric caeca (midgut) and the anus (hindgut) of each fish was removed, weighed aseptically, diluted (10% w/v) in peptone saline solution (0.85% NaCl, 0.1 g peptone), transferred to a stomacher bag and homogenised for 30 seconds in plastic bags by Stomacher® 400 at room temperature. One milliliter of homogenate was used for serial dilutions. One milliliter of each dilution was placed on the bottom of a Petri dish and successively 20 ml of a different molten agar media was poured onto the duplicate and mixed with the inoculum.

Culture media and growth conditions

The count of aerobic or aerotolerant heterotrophic bacteria was determined on Nutrient Agar (NA; Microbiol) after incubation at 28°C for 72h as indicated by Hu *et al.*, 2008. The enumeration of Enterobacteriaceae, Coliforms and *Escherichia coli* was performed on Violet Red Bile Glucose Agar (VRBGA; Microbiol) and Violet Red Bile Agar Mug (VRBA-MUG; Microbiol) kept at 30°C for 24 h, respectively. The cultivation and the count of probiotics were made on de Man-Rogosa-Sharpe Agar (MRS; Microbiol) incubated at 28°C for 7 days. Bacterial counts were expressed as colony forming units per gram ($CFUg^{-1}$). Afterwards, a total of 200 colonies from NA plates were isolated randomly and streaked on fresh media four times to obtain pure culture. The purified isolates were stored at -80°C in a 40% (v/v) glycerol-Nutrient Broth solution. In this study a total of 120 purified bacterial colonies were reactivated on Nutrient Agar medium for collecting the cells to be processed for genetic analyses.

Genetic analysis

Bacterial cell preparation for DNA extraction to be used for PCR amplification and the protocol for the restriction analyses and purification of 16S rDNA amplicons for sequence study were carried out as described by Floris *et al.* (2011b). Partial sequences of two or four 16S rDNA amplicons of the most numerous groups distinguished by restriction analyses were determined by BMR genomics S.r.l, Padova and edited with the software Chromas version 1.43 (Griffin University, Brisbane, Qld, Australia). The results of sequencing were submitted for homology searches by BLAST (Basic Logical Alignment Search Tool; Altschul *et al.*, 1990) after unreliable sequences at the 3' and 5' ends were removed. The data-base used for sequence pairing was that of the NCBI (National Center for Biotechnology Information) http://www.ncbi.nml.nih.gov, The identities were determined on the highest score basis.

Statistical analyses

Data of microbial counts were manipulated using the One-Way ANOVA test in order to compare the number of bacterial groups estimated on different culture media.

Results

Bacterial counts of aerobic or aerotolerant culturable bacteria from the gilthead sea bream's gut were determined using NA medium (Microbiol) as a general medium for studying intestinal microbial ecology. The viable counts registered on intestinal samples from the Tortoli lagoon ranged from 190 to 6,900 $CFUg^{-1}$ while the values estimated for the Porto Pino sea bream varied from 65 to 3,100 $CFUg^{-1}$. As regards the number of Enterobacteriaceae, the scored values were from 50 to 1,145 $CFUg^{-1}$ in the Tortoli fish gut and from 0 to 620 $CFUg^{-1}$ in the Porto Pino samples while Total Coliforms were registered from 30 to 725 $CFUg^{-1}$ and from 0 to 790 $CFUg^{-1}$ on the Tortoli and Porto Pino intestinal tracts, respectively. Moreover, variations in gut bacterial counts between individual fish inside the same group were highlighted in this study although ANOVA did not detect any significant difference between the two groups of sea bream. Considering the counts of probiotics cultivated on MRS medium, the presence of bacteria able to grow on this medium was observed only in one intestinal sample, coming from Tortoli and numbering up to 6 colonies per plate, while no bacteria on MRS were detected when cultivating gut samples of fish from the Porto Pino lagoon.

On the other hand, significant differences were observed in the qualitative composition of the bacterial population associated with the gut of the two groups of gilthead sea

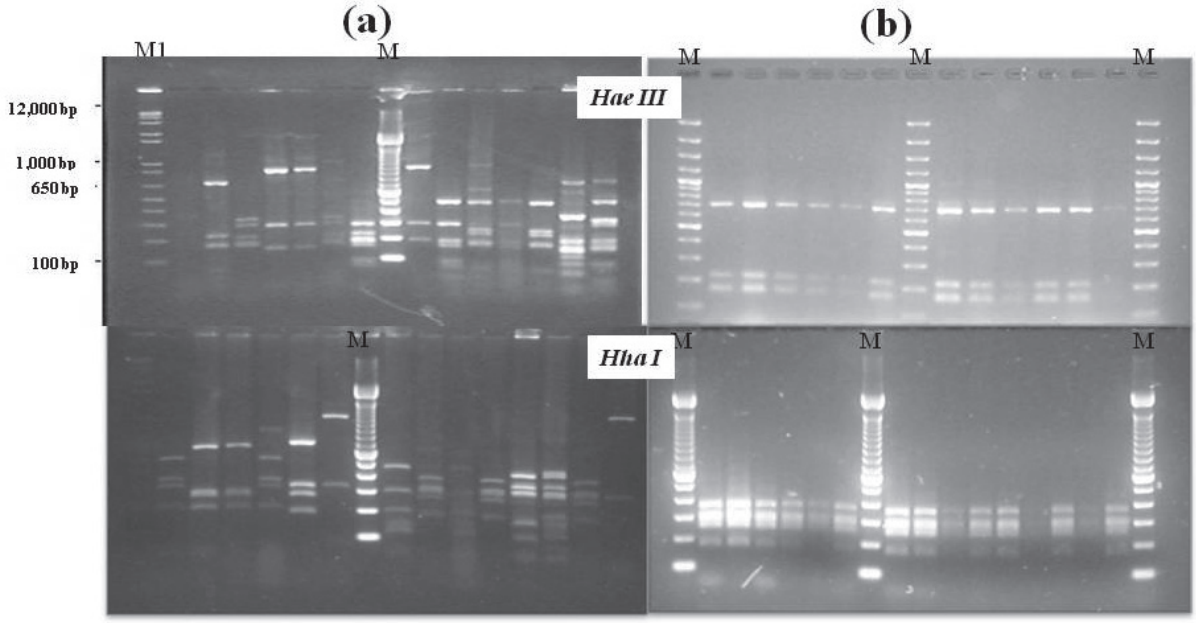

Figure 2. Restriction profiles of 16S rDNA from intestinal bacteria from gilthead sea bream captured in Tortoli (a) and Porto Pino (b) lagoons, run on 2% agarose gel after digestion with *HaeIII* (top) and *HhaI* (bottom) restriction enzymes. M1: 1 Kb Plus DNA ladder Marker Size (100-12,000bp, Invitrogen); M: 100bp DNA Ladder (100-2,072bp, Invitrogen).

bream from different environments. Analysis of the restriction profiles made on the basis of the number and the molecular weight of the bands produced after digestion by *HaeIII* and *HhaI* restriction enzymes, allows us to group the bacteria into different clusters of ribotypes. Examples of ARDRA are shown in Figure 2 (a and b). The number of bands ranges from 2 to 10 and their molecular weights were estimated from 80 to about 1000 bases pair (bp). As for the intestinal bacterial flora of the sea bream from Tortoli, a high diversity was noted with a total of 19 different biotypes on 57 strains while only 3 distinct ribotypes out of 63 isolates were delineated in the gut of the Porto Pino fish, by combining the restriction profiles obtained with the two enzymes. Identification of representative strains of the various ARDRA clusters indicated a diverse microbial composition in the intestinal tract of the gilthead sea bream from the two ecosystems. Figure 3 shows the relative abundance of bacterial genera and species (%) identified on the basis of the highest homology (100%) of partial 16S rRNA gene sequence by BLAST search. The results of sequence analysis on bacteria from the Tortoli fish ascribed them to 13 taxonomic groups including *Pseudomonas* spp. (33.3%), *Sphingomonas paucimobilis* (10.5%), *Proteus* spp. (8.8%), *Chryseobacterium* sp. B-G-R2A3 (5.3%), Arctic soil bacterium A1T3 (5.3%), *Sphingobacterium* spp. (5.3%), *Psychrobacter* spp. (3.5%), *Psychrobacter maritimus* (3.5%), *Leucobacter* spp. (3.5%), *Yersinia bercovieri* (3.5%), *Aeromonas* spp. (3.5%), *Aeromonas molluscorum* (1.7%) and *Erwinia persicina* (1.7%) (Fig. 3a). Furthermore, two amplicons of the same ARDRA group were assigned to the species Arctic soil bacterium A1T3 and *Chryseobacterium* sp. B-G-R2A3 by Blast.

On the other hand, considering the isolates from the Porto Pino gut samples, the three

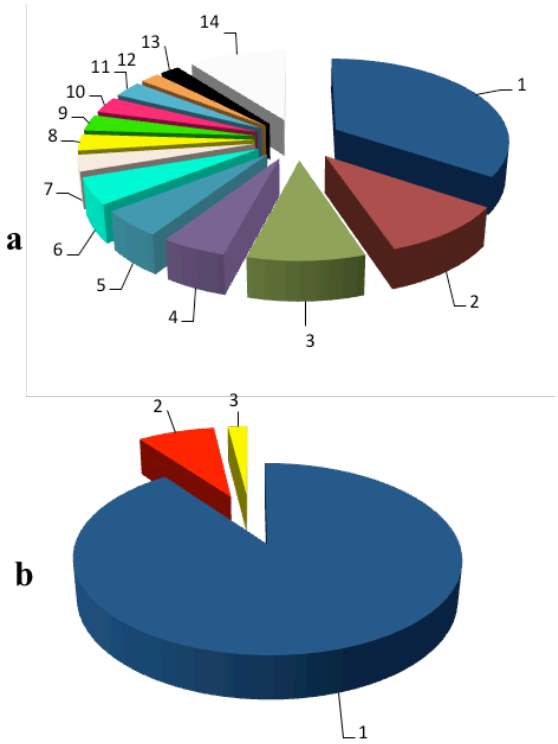

Figure 3. Relative abundance of bacterial genera in the intestinal tract of gilthead sea bream from (a) Tortoli and (b) Porto Pino lagoons. (a) 1, *Pseudomonas* spp. (33.3%); 2, *Sphingomonas paucimobilis* (10.5%); 3, *Proteus* spp. (8.8%); 4, *Chryseobacterium* sp. B-G-R2A3 (5.3%): 5, Arctic soil bacterium A1T3 (5.3%); 6, *Sphingobacterium* spp.(5.3%); 7, *Psychrobacter* spp. (3.5%); 8, *Psychrobacter maritimus* (3.5%); 9, *Leucobacter* spp. 3.5%; 10, *Yersinia bercovieri* (3.5%); 11, *Aeromonas* spp. (3.5); 12, *Aeromonas molluscorum* (1.7%); 13, *Erwinia persicina* (1.7%); 14, Other Gram negative (10.5%). (b) 1, *Pseudomonas* spp. 90.5%; 2, *Janthinobacterium* spp. 7.9%.; 3, *Psychrobacter maritimus* 1.6%.

ARDRA clusters were affiliated into the following taxonomic groupings *Pseudomonas* spp. (90.5%), *Janthinobacterium* spp. (7.9%) and *Psychrobacter maritimus* (1.6%) (Fig. 3b).

Discussion

This work represents a polyphasic study on the cultivable intestinal microflora of gilthead sea bream coming from two coastal lagoons of Sardinia. Bacterial counts registered using different growth media highlight fish-to-fish variation in microbial loads as observed in other studies on the cultivable intestinal bacteria of rainbow trout (Spanggaard *et al.*, 2000). Despite this fact, one-way ANOVA did not reveal any differences between the microbial loads of the two groups of fish and these results were consistent with those estimated for the gut of gilthead sea bream captured during the winter season in two other coastal lagoons of Sardinia (data not shown). As for the absence of bacteria able to grow on MRS medium, the same results were obtained in studies on intestinal microbial communities of rainbow trout (*Oncorhynchus mykiss*) (Kim *et al.*, 2007).

Throughout the present research, the use of 16S rRNA primers, restriction analysis and partial sequencing of the 16S rRNA gene, provided an important means of elucidating the biodiversity of the intestinal microbial community of gilthead sea bream which live in different ecosystems. This study showed that the most abundant cultivable bacteria belongs to *Gammaproteobacteria*, and to a lesser extent to *Alphaproteobacteria*, *Betaproteobacteria*, *Actinobacteria* and *Bacteroidetes*. Indeed, *Pseudomonas* spp. were retrieved in the gut microbiota of all groups of gilthead sea bream and this suggests that *Pseudomonas* spp. can be considered natural and in a certain way prevalent members of the aerobic culturable microbial community of the *Sparus aurata* intestinal tract. This bacterial group was also identified as a component of the cultivable intestinal microflora of *Salmo trutta* (Skrodenyte-Arbaciauskiene *et al.*, 2008), Atlantic herring (*Clupea harengus*) (Curson *et al.*, 2010) and gilthead sea bream reared in *off-shore* floating cages (Floris *et al.*, 2011a). In any case, it is well-documented that *Pseudomonas* spp. are ubiquitous bacteria

in nature in both animal and plant products and represent a heterogeneous phylogenetic microbial group which was found to be a component of the edible part of fish and responsible for the spoilage of gilthead sea bream *Sparus aurata* in Mediterranean Sea waters (Tryfinopoulou *et al.*, 2002).

Considering the microbial composition of the two groups of gilthead sea bream, significant differences were noted, such as the presence of a broad range of bacterial species peculiar to the gut tract of fish from the two lagoons. Moreover, different taxonomic affiliations were attributed to two strains of the same ARDRA cluster among the isolates from the Tortoli samples. These results suggest that biodiversity at species level may actually be greater than was detected in this study. However, the isolation and identification of Arctic soil bacterium A1T3 and *Chryseobacterium* sp. B-G-R2A3 from the gut of sea bream coming from the Tortoli lagoon is quite interesting because these species are described in literature as a large group of psychrotolerant species commonly found in Arctic and Antarctic environments (lakes, coastal lagoons and the surface of marine macroalgae) (Mannisto *et al.*, 2006; Michaud *et al.*, 2008).

In the present study, sequence analysis ascribed to *Aeromonas* spp. some strains from the Tortoli gut samples; this taxonomic group was also found among the microflora of trout (Lee *et al.*, 2002; Pond *et al.*, 2006; Kim *et al.*, 2007), the intestinal microbial community of carp (Namba *et al.*, 2007) and the gut of salmon (Skrodenyte-Arbaciauskiene *et al.*, 2008). Additionally, the occurrence of high GC Gram-positive bacteria belonging to *Leucobacter* spp. (3%), detected as a component of the microflora of gilthead sea bream from the Tortoli lagoon, was in accordance with other studies on the intestine of rainbow trout (Kim *et al.*, 2007).

On the basis of the results presented in this article we can presume that the differences observed in the intestinal microbial ecology of the two groups of gilthead sea bream coming from diverse ecosystems could be mainly attributed to the nourishment of these fish. Indeed, different trophic conditions are documented and under monitoring by ARPA-Sardegna for the two aquatic environments, and data from literature confirms the general influence of feeding intensity and the effect of food composition on species diversity and abundance of microorganisms in fish intestines (Izvekova *et al.*, 2007). In the same way, the environmental factors of the two lagoons and in particular the selective hyperhaline conditions present in the Porto Pino lagoon may have influenced the food chain and pabulum as also observed in other studies on fish growth performances in this ecosystem (Rossi and Cannas, 1982), thus determining a low intestinal microbial diversity. For this reason, the use of growth media containing NaCl could be advisable for cultivating bacteria isolated from and selected by this particular environment.

In any case, the interesting bacterial biodiversity observed in this study appears to deserve further genetic studies, such as strain "typing" in order to analyse the entire bacterial chromosome and the presence of extrachromosomal DNA, for a more in-depth analysis of the genetic biodiversity of these microbial populations.

Conclusions

In the light of the present results, this study provides conclusive evidence that the composition of intestinal microflora changes under the effect of abiotic and biotic factors, the type of feeding and in general the habitat, as affirmed by other authors, and the gut microflora therefore represents a "peculiar" ecosystem, an ecological niche which can represent a possible biomarker of environmental origin. Moreover, the presence of bacterial species "naturally" present in aquatic ecosystems and the good state of

health of the gilthead seabream studied can be interpreted as an index of environmental quality. This is an initial step for studying the intestinal microbial ecology of *Sparus aurata* inhabiting Sardinian coastal lagoons. Further analyses are being conducted on other gilthead sea bream from different marine areas, in diverse seasons by combining both cultural techniques and culture-independent methods for a more in-depth study of the intestinal microbial ecology of *Sparus aurata* specimens. This would help to evaluate the ecological significance of these bacteria as bioindicators.

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