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Full Length Research Paper

Lactic acid bacteria associated with the digestive tract and skin of Sea bream (*Sparus aurata*) cultured in Tunisia

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Thirty-seven (37) enterococcal isolates were recovered from the skin and intestines of the sea bream (*Sparus aurata*), the most economically important fish species of the Mediterranean sea from Tunisian fish farming sites, to investigate their antimicrobial potential. All isolates were identified to the species level using genotypic tools. An investigation employing 16S rDNA sequencing in combination with randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) highlighted the predominance of the *Enterococcus faecium* (46%) and *E. faecalis* (19%) species. Other species, such as *E. sanguinicola* (3 strains), *E. casseliflavus* (3 strains), *E. gallinarum* (2 strains), *Carnobacterium* sp. (1 strain), *Aerococcus viridans* (2 strains) and *Vagococcus carniphilus* (2 strains) were also identified. The susceptibility to different antibiotics in addition to the antibacterial activities were investigated for all species identified. The isolates were sensitive to vancomycin but were resistant to several antibiotics relevant for therapy in human and animal medicine. Antibacterial profiles assayed against 39 bacterial indicators (including food-borne and fish pathogenic bacteria in aquaculture as well as other spoilage bacteria) showed that 46% of the isolates exhibited a large inhibition spectrum mainly towards *Listeria monocytogenes*, *Staphylococcus aureus*, *Aeromonas hydrophila, Aeromonas salmonicida, Vibrio anguillarum* and *Carnobacterium* strains. Therefore, highly inhibitory enterococcal strains could potentially be used as probiotics in sea bream and other farming fish fields.

Key words: Enterococcus, lactic acid bacteria, aquaculture, probiotic, sea bream.

INTRODUCTION

The frequent usage of antimicrobial agents has led to the development of multiple antibiotic resistance (MAR) in bacteria and has reduced the efficacy of antibiotic treatment for human and animal diseases (Tendencia and de la Pena, 2001; Pandiyan et al., 2013). Several studies implicated the use of antimicrobials in the fish farming sector and its environment for the prevention and treatment of animal and plant infections as well as for

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promoting growth (Serrano, 2005; Kümmerer, 2009; Martinez, 2009).

The culture practices for most farmed fish species are mostly semi-intensive or intensive and farms are often affected by widespread antibiotic resistance in pathogens (*Aeromonas hydrophila, Aeromonas salmonicida, Edwardsiella tarda, Edwardsiella icttaluri, Vibrio anguillarum, Vibrio salmonicida, Pasteurella piscida* and *Yersinia ruckeri*), which are currently treated with antibiotics (Ben Kahla-Nakbi et al., 2009). Thus, replacing drugs with effective and inexpensive probiotics is necessary to avoid resistance in fish farming sites and antibiotic residues in fish flesh destined for human consumption (Rengpipat et al., 2008).

Lactic acid bacteria (LAB) belong to the bacterial communities present in the normal intestinal flora of fish and exhibit probiotic properties for aquaculture applications. Previous studies on several fish farming applications have shown the antagonistic properties of LAB on fish pathogens (Gatesoupe 1991; Ringo et al., 1995; Gonzalez et al., 2000; Vijayabaskar and Somasundaran, 2008; Rengpipat et al., 2008).

Within the LAB group, *Enterococcus spp*. are widespread in the gastrointestinal tract of mammals, birds, reptiles, insects and are found in the intestinal contents of several healthy fish species and therefore they could be amended to animal food as probiotics to contribute to the health of farmed fish (Campos et al., 2006; Calo-Mata et al., 2007).

Gilt-head sea bream (*Sparus aurata*), which together with sea bass (*Dicentrarchus labrax*) represent the main fish species with high economic value cultured in Mediterranean aquaculture and the main marine fish farmed in Tunisia, is affected by infectious diseases and the abusive use of antibiotics (Zorrilla et al., 2003; Ben Kahla-Nakbi et al., 2007). Even though it is well known that intestinal microflora, especially LAB, might influence the growth and health of farmed fish, there is no information available to date about the composition of the intestinal microflora in the sea bream that are widely cultured in Tunisia. Thus, the present study was firstly designed to investigate the presence and type of LAB of both the skin and gastrointestinal tract of farmed sea bream and to inquire about their bioactive potential against bacterial pathogens. To do so, we have characterised a large collection of sea bream LAB by phenotypic and genotypic analysis (including 16S rRNA sequencing and RAPD-PCR) and carried out the screening of their antimicrobial susceptibility patterns and their ability to produce antibacterial compounds against spoilage and fish pathogenic bacteria.

MATERIALS AND METHODS

Fish and experimental conditions

Gilt-head sea bream (*S. aurata*) specimens were collected from a fish farm in Hergla (central coast of Tunisia). Fish specimens were sampled in a water-ice mixture and kept in ice for 3 h until they arrived at our laboratory. A total of 30 fish specimens with body weights of 180-220 g were examined. Skin patches (2x1 cm^2) were aseptically excised and the intestinal content was removed by dissecting the fish, removing the intestines (to the pyloric caeca) and squeezing out the contents. The gut contents appeared as faecal matter. All samples were weighed and homogenised for 1 min in sterile plastic bags and a Stomacher (Seward, London, United Kingdom). Homogenates of skin or gut were serially diluted in 0.9% saline solution, and 0.1 ml volumes of appropriate dilutions were spread on the surface of MRS (de Man, Rogosa and Sharpe medium) and M17 plates (Oxoid, Ltd., London, UK). The plates were incubated aerobically for 48-72 h at 30°C, and the isolated colonies with typical characteristics, namely pure white and small (2-3 mm in diameter) with entire margins, were picked from each plate and transferred to MRS broth or M17 broth (Oxoid) for experimental use.

Phenotypic characterisation of the bacterial strains

Pure cultures of all the isolates were subjected to the standard tests: colony morphology, cell morphology, motility, Gram stain and the production of cytochrome oxidase and catalase, fermentation tests of glucose and lactose, H_2S and gas production, the ability to grow at 10°C and 45°C in media (Brain Heart Infusion) containing 6.5% NaCl at pH 9.6 (Schleifer and Kilpper-Bälz, 1984). All the Gram-positive bacteria belonging to the LAB group were further tested by means of miniaturised API 50 CH biochemical tests (BioMérieux, Marcy L'Etoile, France). The results of the identification tests were interpreted using the APILAB PLUS software, version 4.0 (BioMérieux).

Genetic identification of LAB strains

DNA from the LAB was isolated from the pellets formed after spinning 1 ml of overnight cultures in MRS broth at 7500 rpm for 10 min. Each pellet was re-suspended in 180 µl of lysis buffer (20 mM Tris-Cl pH8, 2 mM EDTA, 1.2% triton X-100, 20 mg/ml lysozyme). Each 10 ml of lysis buffer was prepared by mixing 4 ml of lysozyme (10 mg/ml, in bi-distilled water), 4 ml of 50 mM Tris-HCl, 200 µl of 100 mM EDTA, 120 µl of Triton X-100 and 1.68 ml of Milli-Q water. All the reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA). After an incubation step at 37°C for 2 h, 25 µl of proteinase K (10 mg/ml) (Sigma) was added, followed by incubation at 70°C for 30 min. Then, the bacterial DNA was purified from each extract by means of a DNeasy tissue minikit (QIAGEN Inc., Valencia, CA, USA), based on the use of micro-columns. The concentration of purified DNA extract was determined by measuring the fluorescence that developed by using a Quanti-iT kit and a Qubit fluorimeter (Invitrogen).

The genetic characterisation of LAB isolates was performed by a PCR amplification of 16S rDNA using the universal set of primers p8FPL (forward: 5'-AGTTTGATCCTGGCTCAG-3') and p806R (reverse: 5'-GGACTACCAGGGTATCTAAT-3') that yield an 800 bp PCR product (McCabe et al., 1995). The amplification conditions were as follows: a previous denaturing step at 94°C for 7 min was coupled to 30 cycles of denaturation (94°C for 1 min), annealing (55°C for 1 min) and extension (72°C for 1 min) and to a final extension step at 72°C for 15 min. All the amplification assays comprised 100 ng of the template DNA, 25 µl of a master mix (BioMix, Bioline Ltd., London, UK), including the reaction buffer, dNTPs, magnesium chloride and *Taq* DNA polymerase, PCR water (Genaxis, Montigny le Bretonneaux, France), and 5 µl of each oligonucleotide primer to achieve a final volume of 50 µl. All PCR assays were carried out on a MyCycler Thermal Cycler (BioRad Laboratories, Hercules, CA, USA). The PCR products were visualized in 2.5% horizontal agarose (MS-8, Pronadisa, Madrid,

Spain) gels.

Prior to sequencing, the PCR products were purified by means of an ExoSAP-IT kit (GE Healthcare, Uppsala, Sweden). Direct sequencing was performed with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The same primers used for PCR were employed for the sequencing of both strands of the PCR products. The sequencing reactions were analysed in an automatic sequencing system (ABI 3730 XL DNA Analyser, Applied Biosystems) with the POP-7 system. Sequence homologies were searched using the BLAST tool (National Centre for Biotechnology Information). The alignment of the new sequences with other ones present in GenBank was accomplished using the ClustalX software (Larkin et al., 2007). Phylogenetic and molecular evolutionary analyses were conducted with the MEGA software (Kumar et al., 2008), using the neighbour-joining method (Saitou and Nei, 1987) and the Kimura 2-parameter with 1000 bootstrap replicates to construct distance-based trees.

RAPD-PCR reaction

RAPD-PCR was performed using 200 ng of the template DNA and 25 µl of a master mix (BioMix, Bioline Ltd., London, UK), including the reaction buffer, dNTPs, magnesium chloride and Taq DNA polymerase and PCR water (Genaxis, Montigny le Bretonneaux, France) and 14 pmol of M13 (5´-GAGGGTGGCGGTTCT-3´) (Andrighetto et al., 2001) to achieve a final volume of 50 µl. The amplification reactions were performed using a thermal cycler from Applied Biosystems (GeneAmp-PCR System 2700). The following reaction conditions were used: initial denaturalisation at 94ºC for 5 min, followed by 33 cycles at 94ºC for 60 s, annealing at 45ºC for 60 s, extension at 72ºC for 60 s, and final extension at 72ºC for 15 min. Ten µl of the PCR products were separated and visualised using 1.5% horizontal agarose gels (MS-8, Pronadisa, Madrid, Spain) in a solution of 1XTAE buffer (Tris-acetate-EDTA) and ethidium bromide (10 mg/ml) with electrophoresis at 80 V. The 123-bp DNA ladder (DNA LADDERS D 5042, Sigma) was used as a size marker. To check the reproducibility, the PCR assays were performed at least three times each. In each reaction, a tube without the template DNA was included as a negative control.

Microbial sensitivity towards antibiotics

The bacterial sensitivity was determined by the agar diffusion method according to Chabbert (1982) using the following 16 antibiotics selected as representatives of the different classes of antimicrobial agents relevant for therapy in human and animal medicine: vancomycin (30µg), penicillin G (10 UI), amoxicillin (25 µg), oxacilin (5 µg), cefoxitin (30 µg), ceftriaxone (30 µg), streptomycin (10 UI), tobramycin (10 µg), neomycin (30 UI), chloramphenicol (30 µg), tetracycline (30 UI), oleandomycin (15 UI), nitrofurantoin (300 UI), trimethoprim-sulphamide (25 µg), rifampicin (30 µg) and oxolinic acid (30 µg). Five ml of overnight culture in MRS broth, of the LAB strains was spread out on the surface of Mueller-Hinton agar plates (Oxoid). Then, the paper disks that were impregnated with the antimicrobial agents were placed onto the agar plate. After overnight incubation at 20°C, the diameter of the zone of inhibition of bacterial growth around each disk was measured. Based on the zones of inhibition, a qualitative report of “susceptible”, “intermediate” or “resistant” was determined for the tested bacteria according to the French National Guidelines (Comité de l’Antibiogramme de la Société Française de Microbiologie, 1996).

Antibacterial activity of LAB strains

The potential bacteriocin-producing strains were screened against a range of 39 indicator pathogenic and spoilage microorganisms (Table 1). The detection of bacteriocin activity in LAB strains was initially screened by means of a standardised agar disk diffusion method. Briefly, Muller-Hinton (Oxoid) agar plates were seeded with a bacterial lawn of each indicator strain at a 10^5 CFU/ml concentration. Then, extracellular extracts were prepared by centrifugation, at 7,000 rpm for 15 min, of 48-h culture in MRS of each strain and the cell-free extract was sterilized by filtration through 0.22 µm (Millex GS, Millipore, St. Quentin, France). Twenty µl of each LAB strain extracellular extract were placed on 6-mm sterile disks (Oxoid) that had previously been placed on the agar plates. The plates were incubated overnight at 37°C, and the antimicrobial activity was detected by the appearance of translucent halos in the bacterial lawn surrounding the disks. A nisin-producing *L. lactis* strain was included as a positive control for the antimicrobial activity.

RESULTS

Isolation of microorganisms

From the different samples of sea bream, 37 microbial isolates were examined; 11 of these strains were isolated from the skin and 26 strains were from the intestinal content. The physiological and biochemical characteristics of the isolated LAB strains are shown in Table 2. Thus, all the isolates were Gram-positive, catalase-negative, non-motile, non-spore-forming and chain-forming cocci, able to ferment glucose and to grow at 10ºC and 45ºC and in media containing 6.5% NaCl. All the strains produced acid from glucose, fructose, arbutin, esculin, maltose and trehalose, but not from arabinose, inositol, starch, rhamnose, dulcitol, inuline, xylitol, turanose, lyscose, fucose, arabitol, ceto-gluconate, erythritol, xylose or adonitol.

Identification

The genomic DNA of all the isolates was purified and ca. 800-bp fragments of their 16S rDNA were amplified and sequenced. The alignment of the 16S rRNA sequences showed that all the strains exhibited very high homology (≥ 95%) among themselves and with other *Enterococcus* strains deposited in the GenBank database. The results of the alignements allowed the classification of nine intestinal strains as *E. faecium*, seven strains as *E. faecalis*, three strains as *E. casseliflavus*, one strain as *Enterococcus gallinarum*, two strains as *E. sanguinicola,* one strain as *Carnobacterium* sp., two strains as *Aerococcus viridans* and two other strains as *Vagococcus carniphilus*. However, ten enterococci could not be identified to the species level but could only be identified to the genus level (Table 2). From the skin, only four different species were isolated (*E. faecium, E. faecalis, A. viridans* and *Carnobacterium* sp.).

The dendrogram derived from the sequence homology comparison of 16S rRNA gene sequences of isolates with respect to the reference sequences from GenBank is

Table 1. Pathogenic and spoilage indicator microorganisms used to test the antibacterial activities of LAB isolates.

Code	Species	Origin
AmH01	*Aeromonas hydrophila*	ATCC 7966
BaC23	*Bacillus cereus*	ATCC 14893
BaP31	*Bacillus pumilus*	ATCC 7061
BaS05	*Bacillus Subtilis ssp. Spizizenii*	ATCC 6633
BxT01	*Brochotrix thermosphacta*	ATCC 11509
CbD21	*Carnobacterium divergens*	ATCC 35677
CbM01	*Carnobacterium maltaromaticum*	LHICA collection
EbA01	*Enterobacter aerogenes*	ATCC 13048
EbC11	*Enterobacter cloacae*	ATCC 13047
HaA02	*Hafnia alvei*	ATCC 9760
KlOx11	*Klebsiella oxytoca*	ATCC 13182
KlP02	*Klebsiella planticola*	ATCC 33531
KlPn21	*Klebsiella Pneumoniae* ssp. *pneumoniae*	ATCC 10031
Lb30A	*Lactobacillus saerimneri*	LHICA collection
MoM02	*Morganella morganii* ssp. *morganii*	ATCC 8076H
PhD11	*Photobacterium damselae*	ATCC 33539
PrM01	*Proteus mirabilis*	ATCC 14153
PrP11	*Proteus penneri*	ATCC 33519
PrV21	*Proteus vulgaris*	ATCC 9484
PsF12	*Pseudomonas fluorescens*	ATCC 13525
PsFr51	*Pseudomonas fragi*	ATCC 4973
PsG21	*Pseudomonas gessardii*	LHICA collection
SrM53	*Serratia marcescens* ssp. *marcescens*	ATCC 274
SyE21	*Staphylococcus xylosus*	ATCC 35983
SyX11	*Stenotrophomonas maltophilia*	ATCC 29971
StM03	*Staphylococcus aureus*	ATCC 13637
59	*Staphylococcus aureus*	ATCC 9144
4521	*Lysteria monocytogenes*	ATCC 35845
4032	*Lysteria monocytogenes*	NCTC 11994
1112	*Lysteria monocytogenes* 1112	LHICA collection
CI34.1	*Pseudomonas anguilliseptica*	Seabream*
ACR5.1(AS)	*Aeromonas salmonicida*	Turbot*
CI52.1(VCI)	*Vibrio anguillarum*	Seabream*
ACC30.1	*Photobacterium damselae* ssp. *piscida*	Sole*
V62	*Vibrio anguillarum*	Seabream**
VF	*Vibrio anguillarum*	Seabass***
AF	*Aeromonas salmonicida*	Seabass***
V90.11.287(V287)	*Vibrio anguillarum*	Seabass****
AH2	*Pseudomonas fluorescens*	*Lates niloticus*****

*Strains provided by Pr. J. L. Romalde (Spain). ** Strain provided by Pr. G. Breuil (France). ***Strains provided by Pr. J. C. Raymond (France). ****Strains provided by Pr. L. Gram (Denmark).

shown in Figure 1. The dendrogram suggests a close relationship between our isolates and the reference strains. According to this classification, the ten isolates that were identified to the genus level could be assigned to *E. faecium* (8 strains), *E. gallinarum* (1 strain) and *E. sanguinicola* (1 strain).

RAPD-PCR analysis

Further genetic intra-specific characterisation of the isolates was performed by RAPD-PCR analysis with M13 primers. The dendrogram derived from the combination of amplification profiles obtained with primers M13 is

Table 2. Biochemical and phenotypical tests of the LAB isolates

Stain	Organe	Oxydase	Gaz	H2S	Glycerol	L-Arabinose	Ribose	D-Xylose	Galactose	Manose	Sorbose	Mannitol	Sorbitol	a-Methyl-D-Mannosie	a-Methyl-D-Glucoside	N-Acetyl-Glucosamine	Amygdaline	Salicine	Cellobiose	Lactose	Melibiose	Saccharose	Melezitose	Raffinose	Glycogene	Gentiobiose	D-Tagatose	L-Fucose	L-Arabitol	Gluconate	Identification
UPAA5	Skin	-	-	-	-	+	+	-	+	+	-	+	+	-	-	+	+	+	+	+	+	-	-	-	-	+	+	-	-	+	*E. faecium*
UPAA9	Intestine	-	+	+	-	+	+	+	+	+	-	+	+	-	-	+	+	+	+	+	+	+	-	+	-	+	+	-	-	+	*E. cassel.*
UPAA11	Intestine	-	-	-	-	-	+	+	+	+	-	+	-	-	+	+	+	+	+	+	+	+	-	+	-	+	-	-	-	-	*E. faecium*
UPAA13	Intestine	-	-	-	+	-	+	-	+	+	-	+	-	+	-	+	+	+	+	+	-	+	-	-	-	+	-	-	+	-	*E. faecalis*
UPAA21	Intestine	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*E. faecalis*
UPAA22	Skin	+	+	-	-	+	+	-	+	+	+	+	+	-	-	+	+	+	+	+	+	-	-	-	-	+	+	-	-	+	*E. faecium*
UPAA26	Intestine	-	-	-	-	+	+	+	+	+	-	+	-	+	-	+	+	+	+	+	+	+	+	-	-	+	+	-	-	+	*E. faecalis*
UPAA34	Skin	-	-	-	-	+	+	-	+	+	-	+	+	-	-	+	+	+	+	+	+	-	-	-	-	+	+	-	-	+	*E. faecium*
UPAA35	Intestine	-	-	-	-	+	+	-	+	+	-	+	+	-	-	+	+	+	+	+	+	-	-	-	-	+	+	-	-	+	*E. faecium*
UPAA38	Skin	-	-	-	+	-	+	-	+	+	-	+	+	-	-	+	+	+	+	+	-	+	+	-	-	+	+	-	-	+	*E. faecalis*
UPAA39	Intestine	-	-	-	-	+	+	+	+	+	-	-	-	-	-	+	-	+	+	+	+	+	-	-	-	+	+	-	-	-	*E. faecium*
UPAA40	Intestine	-	-	-	-	+	+	-	+	+	-	+	+	-	-	+	+	+	+	+	+	-	-	-	-	+	+	-	-	+	*E. faecium*
UPAA44	Skin	-	-	-	-	+	+	+	+	+	-	+	-	+	-	+	+	+	+	+	+	+	-	+	-	+	+	-	-	-	*E. faecium*
UPAA45	Intestine	-	-	-	-	+	+	+	+	+	-	+	-	+	-	+	+	+	+	+	+	+	-	+	-	+	+	-	-	-	*E. faecium*
UPAA46	Intestine	+	-	-	+	-	+	+	-	+	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	*V. carniphilus*
UPAA49	Intestine	-	+	-	+	+	+	+	+	+	-	+	-	-	-	+	+	+	+	+	+	+	-	+	-	+	-	-	-	-	*E. sang.*
UPAA51	Intestine	+	-	-	-	+	+	+	+	+	-	+	-	-	-	+	+	+	+	+	-	+	-	+	-	+	-	-	-	+	*V. carniphilus*
UPAA52	Skin	-	-	-	+	-	+	-	+	+	-	+	+	-	-	+	+	+	+	-	-	+	+	-	-	+	+	-	-	+	*E. faecalis*
UPAA60	Intestine	-	-	-	+	-	+	-	+	+	-	+	-	+	-	+	+	+	+	+	-	+	-	-	-	+	-	-	-	-	*E. faecium*
UPAA61	Skin	-	+	-	-	-	+	-	+	+	+	+	-	+	-	+	+	+	+	+	+	+	-	+	-	+	-	-	-	-	*E. faecium*
UPAA66	Skin	-	-	-	-	+	+	+	+	+	-	+	-	+	-	+	+	+	+	+	+	+	-	+	+	+	+	-	-	-	*E. faecium*
UPAA68	Intestine	-	-	-	+	-	+	-	-	+	-	+	-	-	-	+	+	+	+	-	-	-	-	-	-	+	-	+	-	-	*A. viridans*
UPAA71	Intestine	+	-	-	+	-	+	-	-	+	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	+	-	+	-	-	*E. sang.*
UPAA72	Intestine	-	-	-	+	+	+	-	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	*E. sang.*
UPAA75	Intestine	-	-	-	+	-	+	-	+	+	-	+	+	-	-	+	+	+	+	+	-	+	+	-	-	+	+	-	-	+	*E. faecalis*
UPAA77	Skin	-	+	+	-	-	+	-	+	+	-	+	-	+	-	+	+	+	+	+	+	+	-	+	-	+	-	-	-	-	*Carnob. sp.*
UPAA82	Intestine	-	-	-	-	+	+	+	+	+	-	+	-	+	-	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	*E. gallinarum*
UPAA85	Intestine	-	-	-	-	-	+	-	+	+	-	+	-	+	-	+	+	+	+	+	+	+	-	+	-	+	-	-	-	-	*E. faecium*
UPAA86	Skin	-	-	-	+	-	+	-	+	+	-	+	+	+	-	+	+	+	+	+	-	+	+	-	-	+	+	-	-	-	*A. viridans*
UPAA87	Intestine	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	*E. faecium*
UPAA89	Intestine	+	-	-	+	-	+	-	+	-	-	+	-	-	-	+	+	+	+	+	-	+	-	-	-	+	-	-	-	+	*E. faecium*
UPAA100	Intestine	-	-	-	+	-	+	-	+	+	-	+	-	-	-	+	+	+	+	+	-	+	-	-	-	+	-	-	-	+	*E. faecalis*
UPAA102	Skin	-	+	+	-	-	+	-	+	+	-	+	-	+	-	+	+	+	+	+	+	+	-	+	-	+	-	-	-	-	*E. faecalis*
UPAA103	Intestine	-	-	-	-	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	-	-	+	+	-	-	-	*E. cassel.*
UPAA104	Intestine	-	-	-	-	+	+	+	+	+	-	+	+	-	-	+	+	+	+	+	+	+	-	+	-	+	+	-	-	+	*E. cassel.*
UPAA106	Intestine	-	-	-	-	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	*E. gallin.*
UPAA110	Intestine	+	-	+	+	-	+	+	-	+	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	*E. faecium*

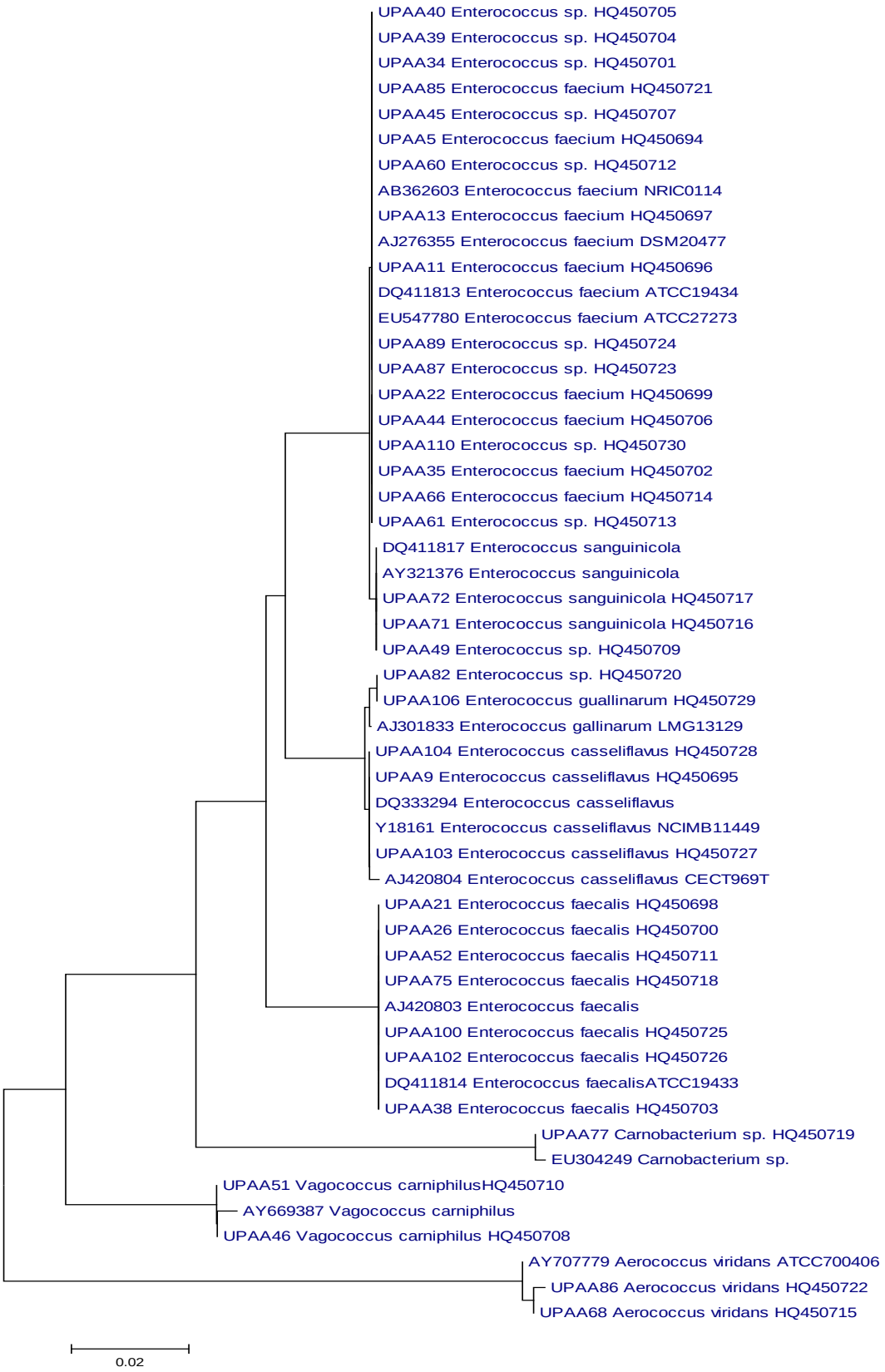

Figure 1. Phylogenetic relationships according to the partial sequencing of the 16S rDNA gene of LAB isolates and reference strains from GenBank by means of the neighbor-joining method. (X) GenBank accession numbers of the LAB isolates.

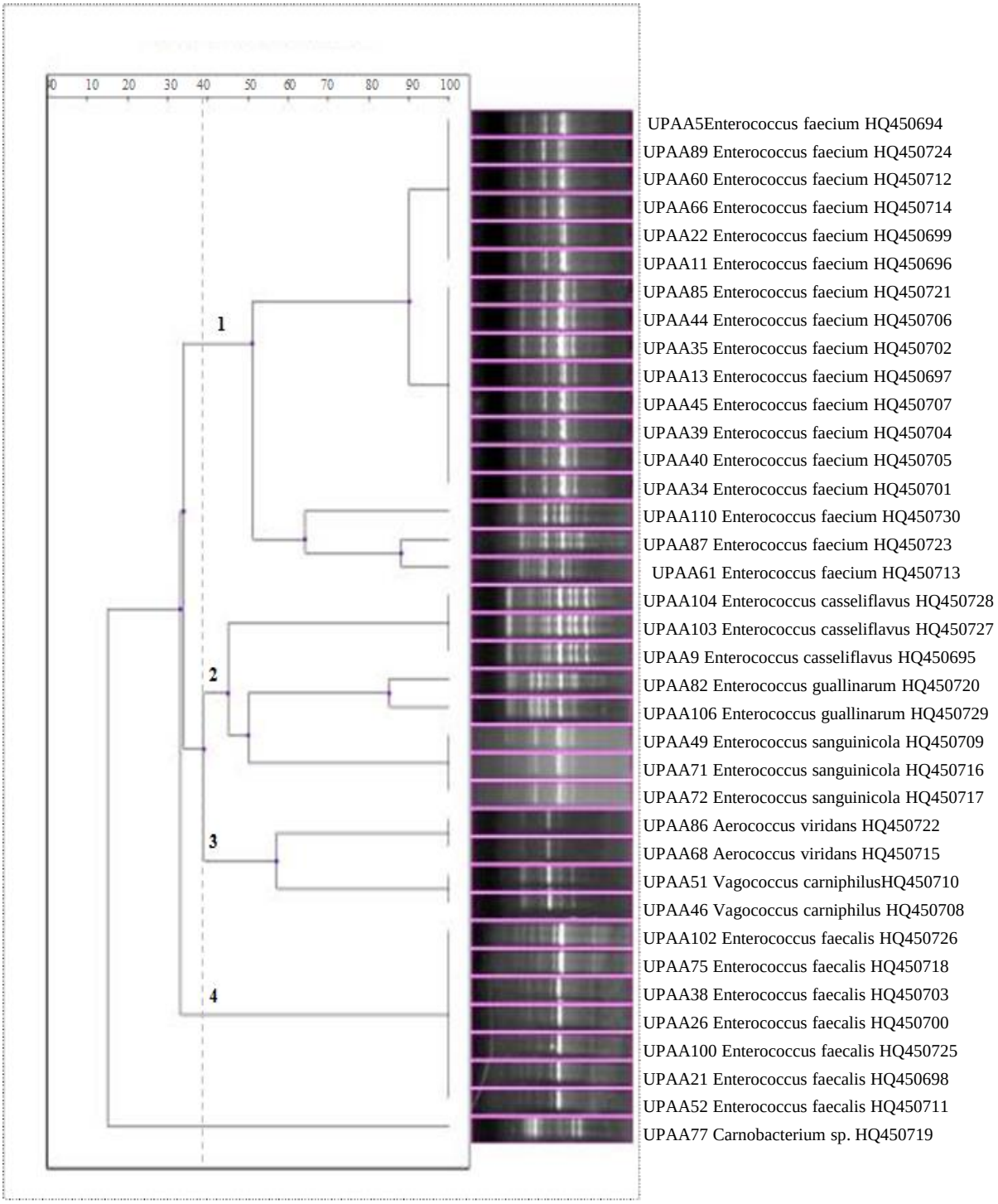

Figure 2. RAPD-PCR patterns of the isolates obtained by using the primers M13, and dendrogram obtained by UPGMA of correlation value of merged normalised RAPD-PCR patterns.

shown as Figure 2. Thus, the RAPD analysis with M13 primers yielded a clear discrimination of the different *Enterococcus* species isolated, allowing their grouping into clusters corresponding to each species. At a similarity level of 40%, arbitrarily chosen for the defining species, four main clusters were observed. The Cluster 1 grouped isolates belonged to the species *E. faecium*. Cluster 2 could be divided into three subclusters, each of them grouping isolates belonging to *E. casseliflavus, E. gallinarum* and *E. sanguinicola*, respectively. The third

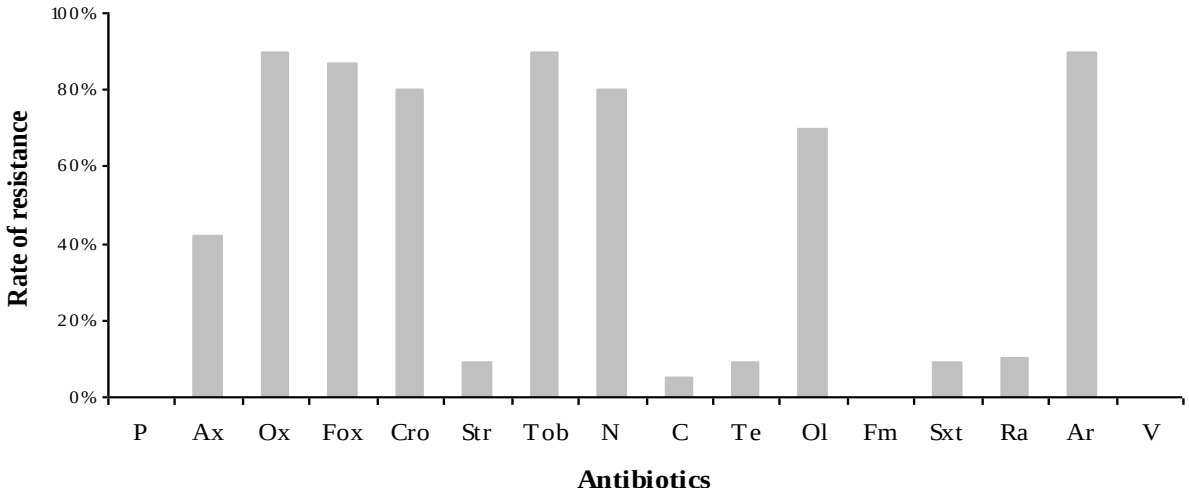

Figure 3. Profiles of resistance obtained for the farmed sea bream LAB strains tested against the 16 antimicrobial agents. P, penicillin; Ax, amoxicillin; Ox, oxacillin; Fox, cefoxitin; Cro, ceftriaxon; Str, streptomycin; Tob, tobramycin; N, neomycin; C, chloramphenicol; Te, tetracycline; Ol, oleandomycin; Fm, furans; Sxt, trimethoprim-sulphamide; Ra, rifampicin; Ar, oxolinic acid; V, vancomycin.

cluster also contained two subclusters that grouped isolates belonging to *A. viridans* and *V. carniphilus.* Finally, a fourth cluster grouped isolates belonging to *E. faecalis* species. The strain *Carnobacterium sp*. clustered as an independent strain. It is particularly interesting to note that the grouping of the isolates with the RAPD analysis was in agreement with the classification provided by 16S RNA sequencing.

After the comparison of the amplification profiles obtained for the isolates that were identified to the genus level with those generated for others strains, it was possible to assign 8 isolates to *E. faecium*, one isolate to *E. gallinarum* and one to *E. sanguinicola,* which confirmed the data provided by the dendrogram generated by 16S RNA sequencing. These results confirmed the data resulting from the dendrogram generated by the 16S RNA phylogenetic analysis.

Microbial sensibility towards antibiotics

All the strains tested were resistant to at least three of the antibiotics. Thus, resistance to oxacillin, cephalosporins (cefoxitin, ceftriaxon), aminoglucosids (tobramycin and neomycin), macrolids (oleandomycin) and oxolinic acid were common among the isolates (Figure 3). In contrast, penicillin, streptomycin, phenicol, tetracyclin, rifampicin, trimethoprim-sulphamid and nitrofurantoin were the most active antibiotics against the majority of the LAB isolates. Nevertheless, it is well known by now that the administration of nitrofurantoin is banned in fish and shellfish farming. Interestingly, all the strains were sensitive to vancomycin.

The resistance patterns of the enterococcal isolates indicated a considerable diversity of strain-specific antibiotypes. Thus, up to 18 different antibiotypes were characterised, including those with resistance to three to ten antimicrobial agents (Table 3). Five different resistance types against seven antibiotics, four resistance types against eight antibiotics, three resistance types against six and nine antibiotics and two resistance types against ten antibiotics were characterised (Table 3).

An analysis of the phenotypic relationships among the enterococci isolated from the skin and intestinal content of the fish was also carried out and showed that, among the 28 antimicrobial resistance patterns obtained, nine were specific to the isolates recovered from the intestines of the fish and that five patterns were specific of those recovered from the fish skin.

Antibacterial activity by LAB isolates

All the isolates were assayed for inhibitory production against 39 Gram-positive and Gram-negative indicator bacteria, including food-borne and fish pathogenic bacteria and other spoilage bacteria (Table1). Seventeen strains (46%) exhibited inhibitory activity against a large number of the indicator strains investigated (Figure 4). Greater inhibition was observed against *L. monocytogenes*, *S. aureus*, *A. hydrophila, A. salmonicida, V. anguillarum* and *Carnobacterium* strains (Table 4). The diameters of the inhibition halos were within the 6.5–20 mm range. Thus, we selected 12 strains that strongly inhibited a large number of indicators and generated inhibitory zones with diameters larger than 11 mm for future studies and to evaluate their potential use as probiotics.

Table 3. Antibiotypes of the LAB strains isolated from farmed sea bream.

Strain	Lab codes	No. of resistance	Type of antimicrobial agents
Enterococcus sp.	UPAA 60	10	N-RA-FOX-TE-OX-CRO-AX-OL-AR-TOB
Enterococcus faecuim	UPAA 66		N-SXT-FOX-OX-CRO-AX-OL-AR-STR-TOB
Enterococcus faecium (6 strains)	UPAA 11/13/22/85/45/61	9	N-FOX-OX-CRO-AX-OL-AR-STR-TOB
Enterococcus faecalis	UPAA 21		N-FOX-OX-CRO-AX-OL-AR-STR-TOB
Enterococcus sanguinicola	UPAA 72		N-FOX-OX-CRO-AX-OL-AR-STR-TOB
Enterococcus faecalis	UPAA 26		N-FOX-TE-OX-CRO-AX-AR-STR-TOB
Enterococcus gallinarum	UPAA 82		N-FOX-TE-OX-CRO-AX-AR-STR-TOB
Enterococcus faecuim (2 strains)	UPAA 89/34		N-FOX-TE-OX-CRO-AX-AR-STR-TOB
Enterococcus faecalis	UPAA 75		N-SXT-RA-FOX-OX-CRO-OL-AR-TOB
Enterococcus gallinarum	UPAA 106	8	N-FOX-OX-CRO-OL-AR-STR-TOB
Enterococcus faecalis (2 strains)	UPAA 102/38		N-FOX-OX-CRO-OL-AR-STR-TOB
Carnobacteruim sp.	UPAA 77		N-FOX-OX-CRO-OL-AR-STR-TOB
Enterococcus faecalis	UPAA 100		FOX-OX-CRO-AX-OL-AR-STR-TOB
Enterococcus faecium	UPAA 5		N-SXT-RA-FOX-OX-OL-AR-TOB
Enterococcus faecalis	UPAA 52		N-FOX-OX-CRO-AX-OL-AR-TOB
Enterococcus faecuim	UPAA 40	7	N-SXT-RA-FOX-OL-AR-TOB
Enterococcus faecuim	UPAA 39		N-OX-CRO-AX-AR-STR-TOB
Enterococcus faecuim (2 strains)	UPAA 44/35		N-FOX-OX-CRO-OL-AR-TOB
Enterococcus casseliflavus	UPAA 104		N-FOX-OX-CRO-OL-AR-TOB
Vagococcus carnophulis (2 strains)	UPAA 46/51		N-FOX-OX-CRO-AR-STR-TOB
Enterococcus faecuim	UPAA 110		N-FOX-OX-CRO-AR-STR-TOB
Enterococcus sanguinicola	UPAA 71		N-FOX-OX-CRO-AR-STR-TOB
Enterococcus sanguinicola	UPAA 49		FOX-OX-CRO-AX-AR-STR-TOB
Enterococcus casseliflavus	UPAA 103		N-C30-OX-AX-OL-AR-STR
Aerococcus viridans	UPAA 86	6	F-C30-OX-AX-OL-TOB
Enterococcus faecuim	UPAA 87		N-FOX-OL-AR-STR-TOB
Enterococcus casseliflavus	UPAA 9		N-FOX-OX-CRO-AR-TOB
Aerococcus viridans	UPAA 68	3	OL-AR-TOB

AX, amoxicillin; OX, oxacillin; FOX, cefoxitin; CRO, ceftriaxon; STR, streptomycin; TOB, tobramycin; N, neomycin; C, chloramphenicol; TE, tetracyclin; OL, oleandomycin; FM, furans; SXT, trimethoprim-sulphamide; RA, rifampicin; AR, oxolinic acid.

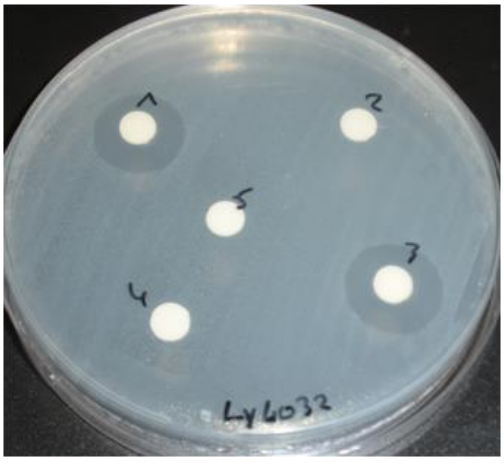

Figure 4. Antimicrobial activity of cell-free supernatants from Enterococcus isolates against *lysteria monocytogenes.* 1, UPAA34; 2, UPAA26; 3, UPAA39; 4, UPAA21 and 5, UPAA13.

DISCUSSION

The high mortality rates that occur in the larval phases of cultures of marine fish such as sea bream (*Sparus aurata*), which is one of the most valuable cultured species in Tunisia and other Mediterranean countries, cause great economic losses to aquaculture facilities in these countries. This mortality has been frequently attributed to bacterial infections (Toranzo et al., 1993; Villamil et al., 2003). Among the possible ways to prevent this problem whilst avoiding the extensive use of antibiotics, is the use of bacteria such as LAB (potential probiotics). This subject has received increasing attention during the last decade (Ringo and Gatesoupe, 1998; Gatesoupe, 1999; Vazquez et al., 2004; Anders et al., 2010). However, to our knowledge no study has described the isolation, screening and characterisation of lactic acid bacteria to be used as probiotics in sea bream or in other fish species from Tunisian aquaculture facilities. Therefore, this study was firstly designed to

Table 4. Antimicrobial activity of the enterococcal isolates against Gram-positive and Gram-negative fish pathogenic and food spoilage micro-organisms.

Producers strain	Indicators strain																								
	AmH01	BaC23	BaP31	BaS05	BxT01	CbD21	CbM01	HaA02	KlPn21	MoM02	PrV21	PsFr51	SrM53	SyX11	59	4521	4032	1112	AS	AF	V62	VCl	V321	V287	VF
UPAA5	7		8	8		10	10	6.5		7		6.5	6.5		9	9	9	8	10		9	10	8		
UPAA11	7	7				10	10		9		8						11	13	10		16	11		10	10
UPAA22	8	7				9	10		9								10	10			10	10		9	
UPAA26	10	7							9		10											10			
UPAA34	8	7	10		6.5	17	10										16	18	10	11	11	10		12	11
UPAA35	9	7	10		6.5	18	12										16	16	10	11	12	11		12	11
UPAA39	8	7	10		6.5	17	12										16	16	10	11	13	10		12	11
UPAA40	8				6.5	14	10										18	18	9	8	15	11		11	8
UPAA44						20	11										18	14	10	10	14	10			
UPAA45																	10	12	10	10	10	10			
UPAA49	9	7	8		6.5												10	10	9	10		9		10	9
UPAA61	7		9	8	6.5					7							9	8	8	9		10			
UPAA71	10	7	10		6.5												11	11	10	10	10	10		11	10
UPAA72	10	7							9		9						10	10	9	9	10	9		9	9
UPAA85	7		8											7			9	9	10	9	12	12			8
UPAA89	7		9											7			10	9	10	10	10	10			10
UPAA110	7			8	6.5		9	6.5		7							13	10	11	10	12	9			

Results are expressed as diameters of the inhibition zone in mm. Indicator strains EbA01, EbC11, KLOX11, KLP02, Lb30A, PhD11, ACC30.1, CI34.1, PrM01, PsF12, PrP11, PsG21, SyE21, StM03 were not inhibited by any LAB strain.

isolate, identify and characterise LAB associated with the skin and intestines of healthy sea bass because these LAB isolated *in situ* are normal residents and are persistent in the skin and intestines of the hosts; therefore, the host immune system should tolerate them (Tannock, 1999).

Remarkably, enterococci were found to be ubiquitous among the fish samples tested. Enterococci are part of the normal intestinal microbiota of humans and animals and are used as indicators of faecal contamination of recreational water, but they can also be isolated from natural environments that have not been contaminated by faecal material (Roberts et al., 2009). Their occurrence in fish and fish environments has been described before (Kanoe and Abe, 1988; Peterson and Dalsgaard, 2003; Michel et al., 2007). The identification of *Enterococcus* species by physiological tests has always been problematic because of their considerable phenotypic diversity (Park et al., 1999), and commercially available kits are frequently insufficient for an accurate identification (Angeletti et al., 2001). Hence, in this work phenotypic analyses were complemented with 16S rDNA phylogenetic analysis and RAPD cluster analysis. A high congruency between RAPD and phylogenetic clusters was observed in this work, which is in agreement with previous reports (Vancanneyt et al., 2002; Linaje et al., 2004). Our work identified *E. faecium* as the most commonly isolated *Enterococcus* species from European sea bream (*Sparus aurata)* (46% of

microbial isolates), followed by *E. faecalis* (19%), and, to a lesser extent *E. sanguinicola*, *E. casseliflavus* and *E. gallinarum*. Three species were also isolated: *Carnobacterium sp*. (one strain), two strains of *Aerococcus viridans* and two strains of *Vagococcus carniphilus.*

E. faecalis, E. faecium and other enterococcal species were not considered as indigenous flora of the fish gut (Ringo and Gatesoupe, 1998). However, Kanoe and Abe (1988) found high counts of *E. faecalis* and *E. faecium* in intestinal samples from marine fish, and Peterson and Dalsgaard (2003) noted the predominance of these two species among the enterococci isolated from integrated and traditional fish farms, suggesting that enterococci may be a member of the normal intestinal flora of fish. The high prevalence of *E. faecium* isolates recovered from our fish intestinal samples support this possibility. Identical results were found when we isolated LAB from a sea bass gut (*Dicentrarchus labrax*) (data not shown). Concerning the genotypic characterisation of the isolates, and as reported in previous studies (Andrighetto et al., 2001; Suzzi et al., 2000; Vancanneyt et al*.*, 2002), RAPD-PCR has been shown to be a valid and accurate method for the identification of enterococci and for detecting genetic diversity at strain level. The results obtained are in agreement with the phylogenetic analysis based on 16S rRNA sequences.

The antibiotic resistance trends among *Enterococcus* species have been extensively reviewed (Bonten et al., 2001; Franz et al., 2003). This matter has been mostly investigated for clinical and human enterococcal isolates because of their high clinical impact. In addition, a number of studies have attempted to compare the resistance spectra of different enterococci according to their human, animal or food origins (Ogier and Serror, 2008). The occurrence of antibiotic resistance among isolates seems to vary somewhat between studies and is often described to be strain- and region-dependent (Canzek et al., 2005) or may also differ according to the isolation method (Klein, 2003).

Enterococci are intrinsically resistant to low levels of penicillin, cephalosporins and aminoglycosides, and currently, these bacteria have acquired high-level resistance to vancomycin and/or aminoglycosides (Roberts et al., 2009). The *Enterococcus* spp. isolated in our study were sensitive to vancomycin, penicillin and nitrofurantoin.

Remarkably, streptomycin, phenicol, tetracyclin, rifampicin and trimethoprim-sulphamid were the most active antibiotics against the majority of the bacterial isolates that were resistant to other antimicrobials tested (oxacillin, cephalosporins, aminoglucosids, macrolids and oxolinic acid). The frequent detection of antibiotic resistance among enterococci is probably due to the increasing use of antibiotics (Bhattacherjee et al., 1988; Pathak et al., 1993; Goni-Urriza et al., 2000; Rhodes et al., 2000), which is complicated by the efficient transfer mechanisms of resistance genes via conjugative plasmids and transposons operating in this bacterial group. Therefore, antibiotic resistance, at least to vancomycin, must be evaluated in these microorganisms before they can be used as probiotics and/or food additives. In our study, all the enterococcal strains tested were sensitive to vancomycin, which is a positive phenotype for selecting these strains as potential probiotics since vancomycin is one of the most clinically relevant antibiotics.

In our study, several bacteria inhibiting strains were selected from both the skin and intestines of the European sea bream (*S. aurata*). Other studies also showed that the skin and gastrointestinal tract of various fish species contain lactic acid bacteria that produce antibacterial compounds able to inhibit the growth of several microorganisms (Ringo 1999; Spanggaard et al., 2001; Rengpipat et al., 2008; Vijayabaskar and Somasundaram, 2008; Ringo, 2008). The antimicrobial spectra on inhibition observed for the *Enterococcus* species included several genera, which indicates a broad spectrum of activity against Gram-positive but also against Gram-negative pathogenic and spoilage organisms.

The fact that these LAB Gram-positive bacteria showed great inhibitory activity towards Gram-negative pathogens is interesting because it is in contrast to the belief that the inhibitory spectrum of LAB is generally restricted to other Gram-positive bacteria (Abee et al., 1995). In agreement with our results, some LAB have been reported to inhibit Gram-negative fish pathogens (Gildberg and Mikkelsen, 1998; Joborn et al., 1997; Ringo, 2008; Robertson et al., 2000). Also, a number of earlier studies have also shown that several marine bacteria produce inhibitory substances that inhibit bacterial pathogens in aquaculture systems (Nogami and Maeda, 1992; Austin et al., 1995; Rengpipat et al., 1998; Gram et al., 1999; Chahad et al., 2007).

Many strains of enterococci, mainly *E. faecalis* and *E. faecium,* are known to produce a variety of bacteriocins active against several pathogenic bacteria, such as *Listeria monocytogenes, Staphylococcus aureus,* and *Vibrio* sp. (Ogier and Serror, 2008). Given their commensal status, enterococci are used as probiotics for humans or farm animals (Tannock and Cook, 2002; Sayyed et al., 2014). The use of such bacteria to inhibit pathogens by the release of antimicrobial substances is now gaining importance in fish farming as a better and more effective alternative to the use of antibiotics to manage the health of these organisms (Vijayan et al., 2006, Iman et al., 2014).

This research has confirmed the abundance of enterococci in European sea bream, both at the skin and intestinal levels and proves that many of the enterococci exhibit inhibitory activity against a number of pathogen and spoilage strains. The selected enterococcal strains described in this study are currently under characterisation to elucidate their potential use as probiotic

bacteria in aquaculture.

Conflict of interests

The authors did not declare any conflict of interest.

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