

To the crazy ones

Here's to the crazy ones.

The misfits

The rebels.

The troublemakers

The round pegs in the square holes

The ones who see things differently.

They're not fond of rules

And they have no respect for the status quo.

You can praise them, disagree with them, quote them,

disbelieve them, glorify them or vilify them.

About the only thing you can't do is ignore them.

Because they change things.

They invent.

They imagine.

They heal.

They explore.

They create.

They inspire.

They push the human race forward.

Maybe they have to be crazy

How else can you stare at an empty canvas and see a work of art?

Or sit in silence and hear a song that's never been written?

Or gaze at a red planet and see laboratory on wheels?

We have the tools for these kinds of people.

Because while some see them as crazy ones, we see genius.

And it's the people who are crazy enough to think they can

Change do world, who actually do.

Anonymus

Aos meus pais, irmãos e sobrinhos
À Joana... o amor da minha vida

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Resumo

Aeromonas salmonicida ssp. *salmonicida*, *Lactococcus garvieae* e *Streptococcus parauberis* são três das principais bactérias patogênicas em aquacultura em Portugal e também a nível mundial, causando problemas quer em animais cultivados quer em animais do meio natural.

Estas três espécies bacterianas foram caracterizadas bioquímica e geneticamente. Os estudos bioquímicos demonstraram uma homogeneidade das suas características, independentemente do método utilizado (clássico ou o sistemas API). No entanto, foi possível observar-se alguma diversidade em alguns dos resultados bioquímicos em todas estas bactérias, particularmente com os sistemas API.

As técnicas de tipagem molecular podem ser ferramentas poderosas quando aplicadas a isolados bacterianos, permitindo a demonstração da existência de uma fonte comum de infecção ou de transmissão da doença. No estudo epidemiológico de isolados bacterianos têm sido aplicadas várias técnicas de tipagem molecular, como por exemplo a análise do conteúdo de plasmídios, Restriction Fragment Length Polymorphism (RFLP), Pulsed-Field Gel Electrophoresis (PFGE), Randomly Amplified Polymorphic DNA analysis (RAPD), Repetitive Sequence-Based Polymerase Chain Reaction (REP-PCR), Enterobacterial Repetitive Intergenic Consensus (ERIC-PCR), e elementos BOX. O PFGE tem sido considerado a melhor técnica para a tipagem de bactérias, no entanto, esta técnica é laboriosa, demorada e tecnicamente difícil de aplicar, o que limita a sua aplicação rotineira em laboratório para a análise de um número elevado de amostras. Os métodos de tipagem molecular baseados na técnica de reacção em cadeia da polimerase (polymerase chain reaction - PCR), como por exemplo RAPD, REP-PCR, ERIC-PCR e BOX, são técnicas rápidas e simples de executar quando comparadas com o PFGE. A amplificação de elementos repetitivos do DNA com base na PCR é normalmente utilizada para a obtenção de padrões de bandas específicos para determinada estirpe bacteriana, que são facilmente analisados com a ajuda de programas de computador específicos.

Com base nestas premissas, as três bactérias de peixe anteriormente referidas foram analisadas por todas ou por algumas destas técnicas de tipagem molecular. Estes estudos de tipagem molecular mostraram a existência de alguma heterogeneidade dentro das três espécies bacterianas analisadas.

A aplicação da técnica de BOX aos isolados de *A. salmonicida* ssp. *salmonicida* permitiu-nos estabelecer grupos baseados na sua origem geográfica, mostrando assim alguma utilidade nos estudos epidemiológicos desta bactéria. Quando aplicamos esta técnica a *L. garvieae* verificamos que também nos foi possível estabelecer grupos baseados na sua origem geográfica. Por sua vez, quando aplicamos a técnica de REP-PCR com o primer (GTG)₅ a isolados de *L. garvieae* foi possível relacionar estes isolados com o local e ano de isolamento. Assim, esta técnica parece ter uma grande utilidade nos estudos epidemiológicos desta espécie bacteriana.

Durante este trabalho foi possível isolar vários fagos patogênicos para *A. salmonicida* ssp. *salmonicida* e *L. garvieae*. Depois de terem sido caracterizados do ponto de vista das suas características biológicas e moleculares, verificou-se que três fagos patogênicos para *L. garvieae* infectavam a quase totalidade das bactérias hospedeiras testadas. No entanto, após estudos moleculares determinou-se que eles eram idênticos.

Apesar de se terem isolado vários fagos patogênicos para *A. salmonicida* ssp. *salmonicida* verificou-se, depois de se realizar o seu host range que nenhum infectava a maioria dos isolados desta bactéria. Assim sendo, decidiu-se não prosseguir com a caracterização destes fagos.

Apesar de durante todo este trabalho se ter realizado um grande esforço no isolamento de fagos contra outras sete bactérias patogênicas para peixes (*Vibrio anguillarum*, *V. ordalli*, *Photobacterium damsela* ssp. *piscicida*, *Tenacibaculum maritimum*, *V. parahaemolyticus*, *Edwardsiella tarda* e *S. parauberis*), nunca nos foi possível isolar fagos contra as mesmas. A única exceção foi *Yersinia ruckeri* para a qual conseguimos isolar dois fagos a partir de água de uma ETAR.

Abstract

Aeromonas salmonicida ssp. *salmonicida*, *Lactococcus garvieae* and *Streptococcus parauberis* are three bacterial pathogens that cause severe health problems in both wild and farmed fish, being responsible for significant economic losses worldwide. Portugal is no exception, with these three species being the most common cause of bacterial fish diseases.

In this study these three bacterial species were characterized, both biochemically and molecularly. The biochemical studies showed that they were all homogeneous, independently if classical or API systems were used. However, all species showed some divergence in the biochemical characteristics, specially in the API systems.

Molecular fingerprinting techniques can provide evidence for a common source of transmission or infection, since they are powerful tools for determining whether strains recovered from different hosts or environments are related.

Several genotyping strategies have been used for epidemiological analysis of bacterial isolates, including analysis of plasmid content, Restriction Fragment Length Polymorphism (RFLP), Pulsed-Field Gel Electrophoresis (PFGE), Randomly Amplified Polymorphic DNA analysis (RAPD), Repetitive Sequence-Based Polymerase Chain Reaction (REP-PCR), Enterobacterial Repetitive Intergenic Consensus (ERIC-PCR), and BOX elements. One method, PFGE, has been considered to be the best strategy for typing bacteria, but the process is laborious, time consuming, and technically demanding, which limits its routine use for processing large numbers of samples. The PCR based molecular typing methods such as RAPD, REP-PCR, ERIC-RPC and BOX are fast and simple to perform, compared with PFGE. Amplification by PCR of the DNA repetitive elements is used to obtain strain-specific DNA fingerprints that can easily be analyzed with pattern recognition computer software.

Based on this premise, the three bacterial fish pathogens previously described (*A. salmonicida* ssp. *salmonicida*, *L. garvieae* and *S. parauberis*) were subjected to all or most of these molecular fingerprinting techniques.

The molecular studies, using different typing techniques (RAPD, REP-PCR, ERIC-PCR and BOX) showed the existence of some heterogeneity. Applying BOX typing to *A. salmonicida* ssp. *salmonicida* we were able to cluster most of the strains accordingly to their geographic origin. In *L. garvieae*, REP-PCR with primer (GTG)₅ is useful for epidemiological studies since it could divide strains accordingly to their place and year of isolation. Also, BOX could have some utility in epidemiological studies of this bacterium since clusters based on their geographic origin could be established.

During this work it was possible to isolate several phages against *A. salmonicida* ssp. *salmonicida* and *L. garvieae*. After studying some of the phages biological and molecular characteristics, we reduced the number of phages against *L. garvieae* to three, which after genomic characterization proved to be the same. Due to the narrow

host range displayed by the phages against *A. salmonicida* ssp. *salmonicida*, their characterization was not pursued.

Even though a huge effort was put in phage isolation against another seven bacterial fish pathogens (*Vibrio anguillarum*, *V. ordalli*, *Photobacterium damsela* ssp. *piscicida*, *Tenacibaculum maritimum*, *V. parahaemolyticus*, *Edwardsiella tarda* and *S. parauberis*), no phages were ever isolated. Only in the case of *Yersinia ruckeri* we were able to isolate a couple of phages from one urban sewage treatment plant.

Keywords

Aeromonas salmonicida ssp. *salmonicida*

Aquaculture

Bacteriophages/phages

Bacterial infections

Bacterial typing

BOX-PCR profiles

Epidemiology

ERIC-PCR - Enterobacterial Repetitive Intergenic Consensus PCR

Lactococcus garvieae

PFGE - Pulsed-Field Gel Electrophoresis

RAPD - Random Amplified Polymorphic DNA

REP-PCR - Repetitive Extragenic Palindromic PCR

Streptococcus parauberis

Palavras Chave

Aeromonas salmonicida ssp. *salmonicida*

Aquacultura

Bacteriófagos/fagos

Epidemiologia

ERIC-PCR - Enterobacterial Repetitive Intergenic Consensus PCR

Infecções bacterianas

Lactococcus garvieae

Perfis de BOX-PCR

PFGE - Pulsed-Field Gel Electrophoresis

RAPD - Random Amplified Polymorphic DNA

REP-PCR - Repetitive Extragenic Palindromic PCR

Streptococcus parauberis

Tipagem bacteriana

Table of Contents

Table of Contents

Resumo	vi
Abstract	ix
Keywords	xii
Palavras Chave	xiv
Table of Contents	xvi
List of Tables	xix
List of Figures	xxi
Abbreviations	xxv
Introduction	1
An overview of bacterial fish pathogens in Portugal	2
Assessment of the distribution of bacterial fish pathogens in a production facility	2
Bacterial load in a sole production facility	4
Isolation of bacterial fish pathogens in Portugal	4
<i>Aeromonas salmonicida</i> ssp. <i>salmonicida</i>	5
<i>Lactococcus garvieae</i>	6
<i>Streptococcus parauberis</i>	10
Application of bacteriophages in aquaculture	12
Objectives	19
Material & Methods	21
1. Sample Collection	22
1.1. Water Collection	22
1.2 - Bacterial isolation	22
1.2.1 – Bacterial isolation from sole	22
1.2.2 - <i>Aeromonas salmonicida</i> ssp. <i>salmonicida</i>	24
1.2.3 - <i>Lactococcus garvieae</i>	25
1.2.4 - <i>Streptococcus parauberis</i>	25
2. Histological sampling and processing	27

3. Biochemical and physiological characterization of the bacterial isolates ..	27
4. Hemolysis Test	28
5. Antimicrobial susceptibility test.....	28
6. Serological identification	28
7. Plasmid isolation	29
8. Extraction of bacterial DNA	29
9. PCR Identification	30
10. Detection of antibiotic resistance genes.....	30
11. Molecular fingerprinting	31
12. Computer data analysis	32
13. Phage isolation and characterization	33
13.1. Bacteria and media	33
13.2. Phage isolation	33
13.3. Phage stocks	34
13.4. Prophage induction	34
13.5. Determination of phage host range.....	35
13.6. Phage purity and genome size determination using PFGE.	35
13.7. Analysis of phage nucleic acids	35
Results	36
Distribution of bacterial fish pathogens in a production facility.....	37
Characterization of Portuguese strains of <i>Aeromonas salmonicida</i> ssp. <i>salmonicida</i>	40
Characterization of Portuguese strains of <i>Lactococcus garvieae</i>	52
Characterization of Portuguese strains of <i>Streptococcus parauberis</i>	66
Phages for treatment of <i>L. garvieae</i> and <i>A. salmonicida</i> ssp. <i>salmonicida</i> infections	77
Discussion	84
Distribution of bacterial fish pathogens in a production facility.....	85
Characterization of Portuguese strains of <i>Aeromonas salmonicida</i> ssp. <i>salmonicida</i>	87
Characterization of Portuguese strains of <i>Lactococcus garvieae</i>	93
Characterization of Portuguese strains of <i>Streptococcus parauberis</i>	98
Phages for treatment of <i>L. garvieae</i> and <i>A. salmonicida</i> ssp. <i>salmonicida</i> infections	101
Conclusions	103
References	106

List of Tables

List of Tables

Table 1 - Pros and cons of bacteriophage therapy (adapted from Oliveira <i>et al.</i> , 2012 and Sandeep, 2006).	16
Table 2 - Place, date and number of isolates of <i>A. salmonicida</i> ssp. <i>salmonicida</i> used in this study and their origin.	25
Table 4 - Date and organ of isolation of the <i>Streptococcus parauberis</i> strains used in this study.	26
Table 3 - Place, date and number of isolates of <i>Lactococcus garvieae</i> used in this study.	26
Table 5 - Place of isolation, number of strains of each bacterial species used in phage isolation work.	33
Table 6 - Number of bacteria (CFU/ml) detected in the water of different places throughout the facility (Figure 1) during the initial sampling period. Numbers represent average values of duplicate counting of CFU/ml in plates with Marine Agar medium. D - P4 was discontinued after the end of July 2009. --- Data not available.	38
Table 7 - Detection of <i>Tenacibaculum maritimum</i> during the present study: numbers of isolates obtained from fish and from water, and results from mucus analysis by PCR.	39
Table 8 - Detection of <i>Tenacibaculum maritimum</i> by PCR in samples of water and sediment/biofilm. + <i>T. maritimum</i> detected; - <i>T. maritimum</i> not detected. PE, T4, T5, T11, T16 and TD – different tanks (numbers in the facility); NT – not tested.	40
Table 9 - Biochemical characteristics of the <i>Aeromonas salmonicida</i> ssp. <i>salmonicida</i> used in this study. Number between brackets represents the percentage of isolates that gave the result. (+) positive result; (-) negative result.	41
Table 10 - Percentage of resistance in the Portuguese strains of <i>Aeromonas salmonicida</i> ssp. <i>salmonicida</i> isolated from different farms.	42
Table 11 - Biochemical characteristics of the <i>Lactococcus garvieae</i> used in this study; (+) positive result; (-) negative result.	53
Table 12 - Date of isolation, number of strains and numerical profiles obtained with the API 20Strep and RAPID 32 Strep systems for the <i>Lactococcus garvieae</i> strains isolated in Portugal, after 24 h incubation.	54
Table 13 - Phenotypic characteristics of <i>Streptococcus parauberis</i> used in this study.	69
Table 14 - Susceptibility of <i>Streptococcus parauberis</i> isolated in Portugal to antimicrobial agents. Between brackets are shown the number of isolates that gave that result.	71
Table 15 - Host range of the different phages isolated against <i>Lactococcus garvieae</i> used in this work: green- complete lysis of the bacterial strain; yellow - incomplete lysis of the bacterial strain; red - resistant bacterial strain.	78
Table 16 - Host range of the different phages isolated against <i>Aeromonas salmonicida</i> ssp. <i>salmonicida</i> used in this work: green- complete lysis of the bacterial strain; yellow - incomplete lysis of the bacterial strain; red - resistant bacterial strain.	79

List of Figures

List of Figures

Figure 1 - Schematic representation of the water flow in the fish farm, location of the filters and sampling points (P1-P9) at the beginning of the study.....	23
Figure 2 - Gas bubbles observed in the body of larval sole.....	37
Figure 3 - The four plasmid profiles obtained with the Portuguese strains of <i>Aeromonas salmonicida</i> ssp. <i>salmonicida</i> , using the Kado & Liu (1981) extraction method. Lane A - 0,05 - 1M basepair (bp) DNA mass marker (Bio-Rad, USA); Lane B - Plasmid profile II; Lane C - Plasmid profile I; Lane D - Plasmid profile IV; Lane E - Plasmid profile III.....	43
Figure 4 - Plasmid profiles obtained with the Portuguese strains of <i>Aeromonas salmonicida</i> ssp. <i>salmonicida</i> , using the Birnboim & Doly (1979) extraction method. Lane A - 0,05 - 1M basepair (bp) DNA mass marker (Bio-Rad, USA); Lane B, C, D, E - plasmid profiles.....	44
Figure 5 - The 11 different DNA fragment profiles, obtained with primer P4 of the Ready-To-Go RAPD Analysis Beads (GE Healthcare, UK) in <i>Aeromonas salmonicida</i> ssp. <i>salmonicida</i> . Lane A - 100 bp Molecular weight ladder (Solis BioDyne, Estonia); Lane B - RAPD type K; Lane C - RAPD type B; Lane D - RAPD type A; Lane E - RAPD type J; Lane F - RAPD type F; Lane G - RAPD type H; Lane H - RAPD type D; Lane I - RAPD type E; Lane J - RAPD type I; Lane K - RAPD type G; Lane L - RAPD type C.	44
Figure 6 - Dendrogram established by Phoretix1D Pro software package (TotalLab, UK) using the Dice similarity coefficient and UPGMA on the basis of the RAPD profiles of <i>Aeromonas salmonicida</i> ssp. <i>salmonicida</i> strains obtained with primer P4 of the Ready-To-Go RAPD Analysis Beads (GE Healthcare, UK).	45
Figure 7 - The eight different DNA fragment profiles, obtained in <i>Aeromonas salmonicida</i> ssp. <i>salmonicida</i> with the REP-PCR technique using the primers REP1R-I and REP2-I. Lane A - 100 bp Molecular weight ladder (Solis BioDyne, Estonia); Lane B - REP type H; Lane C - REP type D; Lane D - REP type G; Lane E - REP type C; Lane F - REP type F; Lane G - REP type B; Lane H - REP type E; Lane I - REP type A.	46
Figure 8 - Dendrogram established by Phoretix1D Pro software package (TotalLab, UK) using the Dice similarity coefficient and UPGMA on the basis of the REP profiles obtained with <i>Aeromonas salmonicida</i> ssp. <i>salmonicida</i> using the primers REP1R-I and REP2-I.....	47
Figure 9 - The four different DNA fragment profiles in <i>Aeromonas salmonicida</i> ssp. <i>salmonicida</i> obtained with the ERIC-PCR technique. Lane A - 100 bp Molecular weight ladder (Solis BioDyne, Estonia); Lane B - ERIC type A; Lane C - ERIC type B; Lane D - ERIC type C; Lane E - ERIC type D.....	48
Figure 10 - Dendrogram established by Phoretix1D Pro software package (TotalLab, UK) using the Dice similarity coefficient and UPGMA on the basis of the ERIC-PCR profiles obtained with <i>Aeromonas salmonicida</i> ssp. <i>salmonicida</i>	49
Figure 11 - The seven different DNA fragment profiles obtained in <i>Aeromonas salmonicida</i> ssp. <i>salmonicida</i> with the BOX technique using primer BOXA1R. Lane A - 100 bp Molecular weight ladder (Solis BioDyne, Estonia); Lane B - BOX type D; Lane C - BOX type C; Lane D - BOX type G; Lane E - BOX type A; Lane F - BOX type E; Lane G - Box type F; Lane H - BOX type B.....	50
Figure 12 - Dendrogram established by Phoretix1D Pro software package (TotalLab, UK) using the Dice similarity coefficient and UPGMA on the basis of the BOX profiles obtained with <i>Aeromonas salmonicida</i> ssp. <i>salmonicida</i> using the primer BOXA1R.....	51
Figure 13 - Rainbow trout with bilateral exophthalmia and rupture of eye, typical signs of <i>Lactococcus garvieae</i>	52
Figure 14 - The 18 different DNA fragment profiles, obtained in <i>Lactococcus garvieae</i> when M13 was applied for RAPD analysis. Lane A, H and M - 100 bp Molecular weight ladder (Solis BioDyne, Estonia); Lane B - RAPD type P; Lane C - RAPD type I; Lane D - RAPD type O; Lane E - RAPD type D; Lane F - RAPD type H; Lane G - RAPD type F; Lane I - RAPD type M; Lane J - RAPD type R; Lane K - RAPD type N; Lane L - RAPD type L; Lane N - RAPD type E; Lane O - RAPD type K; Lane P - RAPD type J; Lane Q - RAPD type G; Lane R - RAPD type Q; Lane S - RAPD type C; Lane T - RAPD type A; Lane U - RAPD type B.....	56

Figure 15 - Dendrogram established by Phoretix1D Pro software package (TotalLab, UK) using the Dice similarity coefficient and UPGMA on the basis of the RAPD profiles obtained with <i>Lactococcus garvieae</i> when using primer M13.....	58
Figure 16 - The 11 different DNA fragment profiles, obtained with <i>Lactococcus garvieae</i> when the REP primer (GTG) ₅ was applied. Lane A, I and N - 100 bp Molecular weight ladder (Solis BioDyne, Estonia); Lane B - REP type C; Lane C - REP type B; Lane D - REP type G; Lane E - REP type E; Lane F - REP type A; Lane G - REP type H; Lane H - REP type F; Lane J - REP type D; Lane K - REP type J; Lane L - REP type K; Lane M - REP type I.....	60
Figure 17 - Dendrogram established by Phoretix1D Pro software package (TotalLab, UK) using the Dice similarity coefficient and UPGMA on the basis of the REP-PCR profiles of <i>Lactococcus garvieae</i> obtained with primer (GTG) ₅	61
Figure 18 - The 12 different DNA fragment profiles obtained with <i>Lactococcus garvieae</i> when primer BOXA1-R was applied. Lane A, H, L, M and Q Molecular weight ladder; Lane B - BOX type H; Lane C - BOX type E; Lane D - BOX type J; Lane E - BOX type D; Lane F - BOX type I; Lane G - BOX type C; Lane I - BOX type F; Lane J - BOX type G; Lane K - BOX type B; Lane N - BOX type K; Lane O - BOX type A; Lane P - BOX type L.....	62
Figure 19 - Dendrogram established by Phoretix1D Pro software package (TotalLab, UK) using the Dice similarity coefficient and UPGMA on the basis of the BOX elements profiles obtained with <i>Lactococcus garvieae</i> strains analysed and obtained with primer BOXA1-R was applied.....	63
Figure 20 - The nine different pusotypes obtained with digestion of <i>Lactococcus garvieae</i> DNA with the restriction enzyme <i>Apal</i> . Lane A - Molecular size marker (<i>Xba</i> I digestion of the DNA of <i>Salmonella</i> Braenderup); Lane B - Pulsotype G type H; Lane C - Pulsotype D; Lane D - Pulsotype B ; Lane E - Pulsotype E; Lane F - Pulsotype F; Lane G - Pulsotype I; Lane I - Pulsotype A; Lane J - Pulsotype H; Lane K - Pulsotype C.....	64
Figure 21 - Dendrogram of <i>Lactococcus garvieae</i> isolates established by Phoretix1D Pro software (TotalLab, UK) based on the Dice similarity coefficient and UPGMA cluster analysis of the nine different <i>Sma</i> I pulsotypes.....	65
Figure 22 - Macroscopic lesions associated with <i>Streptococcus parauberis</i> infection in turbot. (A) Unilateral exophthalmia and eye hemorrhage (arrow), and accumulation of fluid at the dorsal region (*) scale bar = 2 cm. (B) Exophthalmia and periorbital abscesses. Hemorrhagy in the eyes (arrows) and mouth (*) scale bar = 1 cm.	66
Figura 23 - Microscopic lesions associated with <i>Streptococcus parauberis</i> infection in turbot. (A) Dermal <i>stratum spongiosum</i> with Gram-positive cocci. Some bacteria inside inflammatory cells (arrowhead). Some necrotic muscular cells are observed (N) (Gram-Twort) scale bar = 20 µm. (B) Hyperplasia of the meninges (arrowheads) (H&E) scale bar = 200 µm. (C) Hyperplasia of the meninges - dura-mater (arrow) caused by large amounts of bacteria (H&E) scale bar = 10 µm. (D) Meninges with mononuclear inflammatory cells with bacteria (arrows) and some necrosis (PAS) scale bar = 20 µm. (E) Meninges with mononuclear inflammatory cells with bacteria (arrowhead) and some necrosis (Gram) scale bar = 10 µm. (F) Mononuclear inflammatory cells (*) with Gram-positive bacteria in the eye choroid (arrows) (Gram Twort) scale bar = 20 µm. (G) Mononuclear inflammatory cells with Gram-positive bacteria in the liver portal space (arrows), but not inside de blood vessels (Gram-Twort) scale bar = 20 µm (adapted from Ramos et al., 2012).	68
Figure 24 - The 10 different DNA fragment profiles obtained with <i>Streptococcus parauberis</i> strains when subjected to RAPD analysis with primer M13. Lane A, F, H, I, O - 100 bp Molecular weight ladder (Solis BioDyne, Estonia); Lane B - RAPD type D; Lane C - RAPD type G; Lane D - RAPD type F; Lane E - RAPD type H; Lane G - RAPD type B; Lane J - RAPD type E; Lane K - RAPD type J; Lane L - RAPD type C; Lane M - RAPD type I; Lane N - RAPD type A.....	72
Figure 25 - Dendrogram established by Phoretix1D Pro software package (TotalLab, UK) using the Dice similarity coefficient and UPGMA obtained with <i>Streptococcus parauberis</i> strains when subjected to RAPD analysis with primer M13.	73
Figure 26 - The 10 different DNA fragment profiles obtained with <i>Streptococcus parauberis</i> strains when subjected to REP-PCR analysis with primer (GTG) ₅ . Lane A, F, L, O - 100 bp Molecular weight ladder (Solis BioDyne, Estonia); Lane B - REP type D; Lane C - REP type J; Lane D - REP type I; Lane E - REP type E; Lane G - REP type G; Lane J - REP type F; Lane K - REP type C; Lane L - REP type H; Lane M - REP type A; Lane N - REP type B.....	74

Figure 27 - Dendrogram established by Phoretix1D Pro software package (TotalLab, UK) using the Dice similarity coefficient and UPGMA on the basis of the REP-PCR profiles Obtained with <i>Streptococcus parauberis</i> when using primer (GTG) ₅	75
Figure 28 - Dendrogram established by Phoretix1D Pro software package (TotalLab, UK) using the Dice similarity coefficient and UPGMA on the basis of the BOX elements profiles obtained with <i>Streptococcus parauberis</i> when using primer BOXA1-R.....	76
Figure 29 - Pulsed filed gel electrophoresis of the genome size of the three phages against <i>Lactococcus garvieae</i> . Lane A - MidRange I PFG Marker (New England Biolabs, USA); Lane 2 - Phage PPC20.1; Lane C - Phage PPC 31.2; Lane D - Phage QS 24.1.....	82
Figure 30 - Restriction pattern of phage PPC20.1. Lane A - Molecular weight marker; Lane B - Restriction fragments of enzyme <i>Sma</i> I; Lane C - Restriction fragments of enzyme <i>Apa</i> I; Lane D - Restriction fragments of enzyme <i>Xho</i> I; Lane E - Restriction fragments of enzyme <i>Xba</i> I.....	83
Figure 31 - Schematic representation of the water flow through the facility (arrows), location of the filters (new filters are in blue) and sampling points (P1-P9) after redesign of the water flow. Numbers near the sampling points represent the bacterial load (CFU/ml) in plates of Marine Agar.....	86

Abbreviations

ADH - Arginine dihydrolase
αGAL - α-galactosidase
AFLP - Amplified Fragment Length Polymorphism
AMD - Starch
APPA - Alanine phenylalanine proline arylamidase
ARA - Arabinose
βGAL - β-Galactosidase
βGLU - β-Glucosidase
βGUR - β-glucuronidase
βMAN - β-Mannosidase
βNAG - N-acetyl-β-glucosaminidase
CDEX - α-Cyclodextrine
DARL - D-Arabitol
ERIC-PCR - Enterobacterial Repetitive Intergenic Consensus PCR
ESC - Esculin
GTA - Glicil triptophane arylamidase
GLYG - Glycogen
HIP - Hippuric acid
INU - Inulin
LAC - Lactose
LAP - L-leucine-2-naphthylamide
LARA - L-arabinose
MAL - Maltose
MAN - Mannitol
MβDG - Methyl-β-D-glucopyranoside
MLZ - Melezitose
MEL - Melibiose
PAL - Alkaline phosphatase
PCR - Polimerase Chain Reaction
PFGE - Pulsed-Field Gel Electrophoresis
PUL - Pululan
PYRA - Pyrrodidonyl arylamidase
RAF - Raffinose
RAPD - Random Amplified Dolymorphic DNA
REP-PCR - Repetitive Extragenic Palindromic PCR
RFLP - Restriction Fragment Length Polymorphism
RIB - Ribose
SAC - Sucrose
SOR - sorbitol
TRE - Trehalose

VP - Voges-Proskauer

URE - Urease

Introduction

An overview of bacterial fish pathogens in Portugal

Fresh and salt water aquaculture has undergone significant development in Portugal since its integration in the European Union in 1986 (Gouveia, 1990; Gouveia, 1994). This development started in fresh water and thereafter expanded to salt water production. With the intensification of aquaculture, the appearance of infectious diseases, especially those of bacterial etiology, became a major concern due to the serious economic losses they cause. However, in Portugal, the increase in bacterial epizootics was not followed by studies related to their identification and characterization; the first reports of bacterial fish pathogens date back to 1986 (Eiras & Saraiva, 1986; Machado-Cruz *et al.*, 1986), where two different pathogens were isolated (*Pseudomonas fluorescens* and *Plesiomonas shigelloides*). In 1987, a study concluded that no notifiable bacterial or parasitic fish disease could be found in Portuguese trout farms (Eiras *et al.*, 1987; Eiras *et al.*, 1988). In 1989, Saraiva *et al.*, report the first occurrence of *A. salmonicida* ssp. *salmonicida* in Portugal. After this, only in 1996 a new report related to bacterial fish pathogens emerged (Sousa, 1996). In this and other studies from the same author (Sousa *et al.*, 1994; Sousa *et al.*, 1997) *Aeromonas salmonicida* and *Yersinia ruckeri* were isolated from Portuguese fish farms and characterized. In 1996 appears the first report of *Pasteurella piscicida* (*Photobacterium damsela* ssp. *piscicida*) in Portugal (Batista *et al.*, 1996).

Regardless of the inclusion in some works of Portuguese isolates of different bacterial species there are few studies related to bacterial fish pathogens in Portugal. From the work we have done since 1998, when we started supporting Portuguese fish farmers, some isolated studies have emerged (Ramos, 2006; Marques, 2010; Mendes, 2010; Ramos *et al.*, 2012). Outside our group, and to our knowledge, only three different studies regarding bacterial fish pathogens isolated in Portugal have been published (Afonso *et al.*, 2002; Santos *et al.*, 2002; Pereira *et al.*, 2004). However, no overview work of the Portuguese bacterial aquaculture diseases has been done since the original work of Eiras *et al.* (1987, 1988).

Assessment of the distribution of bacterial fish pathogens in a production facility

The expansion of intensive aquaculture has led to increased disease susceptibility of farmed fish and control of the various bacterial fish pathogens has become increasingly difficult. Even though the first report of a bacterial fish pathogen dates back to 1718, almost 300 years ago, very little is still known about the way these bacteria infect fish

and the way they propagate and are able to survive in the environment. Since fish live in water it seems logical to conclude that bacteria pathogenic to fish are able to survive and replicate within the water. Consequently, on a fish farm there are very few ways by which fish can be infected: from the water source or from the introduction of carrier fish (symptomatic or asymptomatic) in the facility. Another way that pathogenic bacteria can be introduced into a fish farm is by the feed supplied to the animals, as was shown for *Streptococcus parauberis* (Toranzo *et al.*, 1994; Romalde *et al.*, 1996a).

After the introduction of a bacterial disease on a fish farm, the infection of new animals occurs mainly by one of three processes: transmission of bacteria by direct contact with diseased fishes; shedding of large amounts of bacteria by diseased animals and subsequent infection of healthy animals; vertical transmission, if the broodstock becomes infected.

Bacteria have the ability to colonize all surfaces. The less smooth a surface is, the higher the probability of bacterial colonization. This general principle also applies to pathogenic bacteria. When bacteria colonize a surface there is a good chance that a biofilm will develop. A biofilm is an aggregate of bacteria that adhere to each other on a surface, and are embedded within a self-produced matrix of extracellular polymeric substance. This matrix protects the cells inside the biofilm while at the same time facilitates communication among the bacteria through biochemical signals. The bacteria living in a biofilm usually have significantly different properties from the free-living bacteria of the same species. This happens because the dense and protected environment of the biofilm allows them to cooperate and interact in various ways. One of the benefits of this environment is increased resistance to detergents and antibiotics, because the dense extracellular matrix and the outer layer of the bacterial cells protect the interior of the community. It has been demonstrated that in some cases antibiotic resistance can increase by a thousand fold (Stewart & William, 2001). One of the most important stages of the biofilm life cycle is the dispersal stage, where cells from the biofilm colony disperse into the environment, allowing the bacteria to spread and colonize new surfaces. The dispersal stage is very important because it can lead to the re-infection of surfaces and animals. All this is particularly true for biofilters - filtration systems based on using living material to capture and biologically degrade pollutants that, in the case of an aquaculture facility, can come from the water supply or are naturally produced by the fish in the facility. It is known that biofilters, if not effectively controlled, can act as re-infection sites for infectious bacteria.

Due to all these constraints, in a working aquaculture facility, pipes, instruments, water, biofilms and fish should be subjected to a regular control regarding bacterial pathogens. Also, there should be regular evaluation of the efficiency of the filter systems in use at the installation such as sand filters, biofilters, UV light and ozone systems, to determine if these systems are working properly.

Bacterial load in a sole production facility

In 2010, as part of a project aimed at intensifying the production of sole (*Solea senegalensis*) by A. Coelho & Castro (ACC) a microbiological study was undertaken to evaluate, at different places on a fish farm, the microbial load of water, the disinfection efficiency of the different filters in the system, and the distribution of *Tenacibaculum maritimum*, the only fish pathogen already known to be present in the farm.

A. Coelho & Castro (ACC) was the only Portuguese company, and one of the few in the world with a closed water cycle for the production of sole. From 2004 until 2007, the company succeeded in expanding their production of this valuable fish species from a pilot-scale to industrial-scale. In order to consolidate their growth strategy, ACC was engaged in the process of significantly increasing the production of sole. In this facility, most of the production was sole, but turbot (*Scophthalmus maximus*) and European sea bass (*Dicentrarchus labrax*) were also reared.

The elevated density of fish needed to increase production raises the threat of disease outbreaks, which often cause high mortality rates and heavy economic losses. Thus, as part of this project a thorough microbiological survey was done in the facility in order to assess, and if possible, improve the health status of the fish.

Knowledge of bacterial infections in sole is limited since its industrial production is fairly recent, and consequently the bacterial species searched for in this work are related to the possibility of cross-contamination from the other species produced in the facility, or those carried by the incoming water used in the facility.

Isolation of bacterial fish pathogens in Portugal

During the last 15 years several different bacterial fish pathogens were isolated in our laboratory from epizootic outbreaks from fish species raised in fresh and salt water, mainly in northern Portugal. These bacterial pathogens were identified as belonging to *Aeromonas salmonicida* ssp. *salmonicida*, *Aeromonas hydrophilla*, *Yersinia ruckeri*, *Lactococcus garvieae*, *Vibrio anguillarum*, *Vibrio* sp., *Streptococcus parauberis*, *Tenacibaculum maritimum*, *Photobacterium damsela* ssp. *piscicida*, *Mycobacterium marinum* and *M. chelonae*, however no biochemical or molecular characterization was initially performed. From all these pathogenic bacteria, in this work three of these species (*A. salmonicida* ssp. *salmonicida*, *S. parauberis* and *L. garvieae*) were biochemically and molecularly characterized.

Aeromonas salmonicida ssp. *salmonicida*

Aeromonas salmonicida ssp. *salmonicida* is considered to be responsible for outbreaks of furunculosis in salmonid production worldwide, being enzootic in geographic areas where susceptible fish are farmed. This disease causes significant economic losses, and is the main reason for the intensive vaccination programs in salmonid mariculture (Bernoth *et al.*, 1997). Despite all the scientific knowledge about this bacterium, the origin and epizootiology of this pathogen still remains unclear, and it has been argued whether the microorganism was introduced in Europe from North America, or vice versa (Bernoth *et al.*, 1997).

Furunculosis was the first bacterial disease described in Portuguese fish farms (Saraiva *et al.*, 1989) and since this initial report several outbreaks have been described (Sousa, 1996; Sousa *et al.*, 1997; Ramos, 2006).

Due to the high homogeneity of *A. salmonicida* ssp. *salmonicida*, epizootiological differentiation based in phenotypic systems, such as biotyping or serotyping, have had very limited success within this bacterial subspecies (Dalsgaard *et al.*, 1994; Austin & Austin, 2007; Cipriano & Austin, 2011). The only system that yielded some success was phage typing (Popoff, 1971a; Popoff, 1971b; Rodgers *et al.*, 1981), but it has not been applied widely.

Genotypic methods such as ribotyping (Nielsen *et al.*, 1994; Sousa, 1996) and plasmid profiling (Bast *et al.*, 1988; Belland & Trust, 1988; Nielsen *et al.*, 1993; Hänninen *et al.*, 1995; Pedersen *et al.*, 1996) have also been used with limited success, although they can be of some help in specific geographical areas (Nielsen *et al.*, 1993; Nielsen *et al.*, 1994). It has been found that this bacterium can carry multiple plasmids of various sizes: small, multi-copy plasmids ranging from 1 to 6 kb, and larger low-copy plasmids from 11 to 150 kb. However, most strains of *A. salmonicida* ssp. *salmonicida* characteristically carry three small plasmids of 5.0, 5.2, and 5.4 kb. Some of the large plasmids are known to carry antibiotic resistance genes, but other than this no correlation can be drawn between plasmid carriage and virulence (Brown *et al.*, 1997).

Genome polymorphism studies based on DNA-fingerprinting methods are useful for taxonomic and epizootiological studies of bacterial pathogens. Several methods have been applied to *A. salmonicida* ssp. *salmonicida* such as DNA:DNA reassociation (Belland & Trust, 1988), DNA probes (Gustafson *et al.*, 1992), restriction endonuclease analysis (McCormick *et al.*, 1990), Random Amplified Polymorphic DNA analysis (RAPD) (Hänninen *et al.*, 1995; Miyata *et al.*, 1995; O'hici *et al.*, 2000), Repetitive Extragenic Palindromic (REP-PCR) (Beaz-Hidalgo *et al.*, 2008), Enterobacterial Repetitive Intergenic Consensus (ERIC-PCR) (Beaz-Hidalgo *et al.*, 2008) and restriction fragment length polymorphism analysis by Pulsed-Field Gel Electrophoresis (PFGE) (Hänninen *et al.*, 1995; Chomarat *et al.*, 1998; Livesley *et al.*, 1999; Garcia *et al.*, 2000; O'hici *et al.*, 2000; Giraud *et al.*, 2004). ERIC-PCR and REP-PC showed a great homology among the isolates with similarity values higher than 98 % (Beaz-

Hidalgo *et al.*, 2008). RAPD proved to be more discriminative, differentiating three genetic groups, although they could not be related to the host or geographic origin of the strains (Beaz-Hidalgo *et al.*, 2008). Similar results were obtained with the PFGE technique, showing all *A. salmonicida* ssp. *salmonicida* strains having similarity values higher than 90 % (O'hici *et al.*, 2000). Therefore, although some genetic variability exists within *A. salmonicida* ssp. *salmonicida*, the molecular techniques used until now are not entirely applicable as epidemiological tools.

Antimicrobial therapy has been widely used for controlling outbreaks of furunculosis in cultured fish and the increased use of chemotherapeutics has led to the development of antibiotic resistance in *A. salmonicida* ssp. *salmonicida* (Aoki *et al.*, 1983; Griffiths & Lynch, 1989; Inglis *et al.*, 1991).

The aim of this work was to characterize strains of *A. salmonicida* ssp. *salmonicida* isolated from disease outbreaks in Portugal according to physiological, biochemical, serological and genetic variability.

Lactococcus garvieae

Lactococcosis is an emerging disease caused by *Lactococcus garvieae*, one of the major Gram-positive cocci pathogenic for fish. This bacterium causes high mortalities and huge economic losses. *L. garvieae* was described in 1983 when it was first isolated from a cow mastitis in the United Kingdom (Collins *et al.*, 1983). Contrary to its limited importance in cow mastitis, this bacterium has become one of the most important risk factors in trout production worldwide, especially in the European trout industry, where it has caused losses with up to 90 % mortalities (Pereira *et al.*, 2004). The story of how this bacterium became such a problem for aquaculture is an interesting example of how a pathogen settles in a new niche and how modern aquaculture influences the spread of pathogens.

In Japan, Gram-positive cocci have caused diseases in fish for over 50 years (Hoshima, 1956). However, only in 1991, when strains collected during 1979 from diseased fishes were analyzed, was it possible to establish the association between *L. garvieae* and infected fish. These strains were described as *Enterococcus seriolicida* (Kusuda *et al.*, 1991), and in subsequent studies it was demonstrated that this bacterium was a junior synonym of *L. garvieae* (Doménech *et al.*, 1993; Eldar *et al.*, 1996).

The first infection of *L. garvieae* in trout was recorded in Spain in 1991 (Doménech *et al.*, 1993), and again in 2001 (Ravelo *et al.*, 2001). From here, the spread of the bacterium throughout southern Europe was rapid; in 1992 the pathogen was detected in Italy (Guittino & Prearo, 1992) and again in 1996 and 1999 (Eldar *et al.*, 1996; Eldar & Ghittino, 1999; Eldar *et al.*, 1999), England (Bark & McGregor, 2001; Algöet *et al.*, 2009), Turkey (Diler *et al.*, 2002; Altun *et al.*, 2004; Çağırman, 2004; Altun *et al.*, 2013),

Bulgaria, France and the Balkans (Eyngor *et al.*, 2004), Portugal (Pereira *et al.*, 2004), Greece (Eyngor *et al.*, 2004; Savvidis *et al.*, 2007), Belgium and Switzerland (Algöet *et al.*, 2009). Outside Europe, there are reports of *L. garvieae* in countries such as Israel (Eldar & Ghittino, 1999), Iran (Soltani *et al.*, 2008; Sharifiyazdi *et al.*, 2010; Raissy & Ansari, 2011), South Africa (Carson *et al.*, 1993; Schmidtke & Carson, 2003), Australia (Carson *et al.*, 1993; Eldar *et al.*, 1999; Schmidtke & Carson, 2003) and again Japan (Maki *et al.*, 2008).

Outside Europe, *L. garvieae* infections are not limited to trout, with existing descriptions of infection by this bacterium in several species from the genus *Seriola* (yellowtail, amberjack and kingfish) in Japan (Kusuda *et al.*, 1991; Kawanishi *et al.*, 2005), Nile tilapia (*Oreochromis niloticus*), pintado (*Pseudoplathystoma corruscans*) in Brazil (Evans *et al.*, 2009), Grey mullet (*Mugil cephalus*) (Chen *et al.*, 2002), Japanese eel (*Anguilla japonica*), tilapia, yellowfin seabream (*Acanthopagrus latus*) in Taiwan (Tsai *et al.*, 2012), and several feral fish species (Colorni *et al.*, 2003; Tsai *et al.*, 2012).

The rapid spread of *L. garvieae* throughout the world may have been a result of multiple routes of dissemination and transmission. In these routes we can include the direct spread through movement of infected fish or asymptomatic carriers, as well as horizontal transmission by contaminated water (Múzquiz *et al.*, 1999; Vela *et al.*, 2000; Afonso *et al.*, 2002).

Besides fish, *L. garvieae* has also been isolated from cows and water buffaloes (Collins *et al.*, 1983; Teixeira *et al.*, 1996; Vela *et al.*, 2000; Vianni & Lázaro, 2003; Kawanishi *et al.*, 2006), bovine and caprine dairy products (Fortina *et al.*, 2007; Foschino *et al.*, 2008; Alrabadi, 2012), pigs (Kawanishi *et al.*, 2006; Tejedor *et al.*, 2011), cats, dogs and horses (Kawanishi *et al.*, 2006), dolphins (Evans *et al.*, 2006), bullfrogs (*Rana catesbeiana*) (Tsai *et al.*, 2012), freshwater prawns (*Macrobrachium rosenbergii*) (Chen *et al.*, 2001), fish parasites (Madinabeitia *et al.*, 2009) and even terrestrial plants (Kawanishi *et al.*, 2007). To complicate things further this pathogen has a low virulence in human infection, but the number of case reports of *L. garvieae* infection from various human clinical specimens is increasing (Elliott *et al.*, 1991; Fefer *et al.*, 1998; James *et al.*, 2000; Mofredj *et al.*, 2000; Vinh *et al.*, 2006; Wang *et al.*, 2007; Li *et al.*, 2008; Teixeira *et al.*, 2009; Chan *et al.*, 2011; Zuily *et al.*, 2011). All of these facts indicate the expanding importance of *L. garvieae*.

In trout, *L. garvieae* infection is a hemorrhagic septicemia that occurs throughout the year, but mortalities are higher during the summer months, when water temperature is above 15 °C (Doménech *et al.*, 1993; Pereira *et al.*, 2004; Vendrell *et al.*, 2006; Sharifiyazdi *et al.*, 2010; Austin & Austin, 2012). In trout, typical clinical signs of the disease are quite similar to those described in other fish species like yellowtail or grey mullet (Kusuda *et al.*, 1991; Chen *et al.*, 2002), and they include melanosis, lethargy, loss of orientation, erratic swimming, exophthalmia (uni- or bilateral), periocular and intraocular hemorrhages, accumulation of ascitic fluid in the peritoneal cavity which

may be purulent or may contain blood and strong congestion of the internal organs. The main organs affected are spleen, liver, brain, gut, kidney and heart (Doménech *et al.*, 1993; Pereira *et al.*, 2004; Vendrell *et al.*, 2006; Sharifiyazdi *et al.*, 2010; Austin & Austin, 2012).

Several studies have phenotypically characterized and tried to demonstrate the heterogeneity of *L. garvieae* strains isolated from different animals and countries, using conventional methods and miniaturized systems (API 20Strep, RAPID32 Strep and API 50CH) (Collins *et al.*, 1983; Elliott *et al.*, 1991; Eldar *et al.*, 1999; Vela *et al.*, 2000; Chen *et al.*, 2001; Ravelo *et al.*, 2001; Colorni *et al.*, 2003; Altun *et al.*, 2004; Çağırman, 2004; Pereira *et al.*, 2004; Evans *et al.*, 2006; Kawanishi *et al.*, 2007; Tejedor *et al.*, 2011; Altun *et al.*, 2013). In some of these studies, biotyping schemes were proposed, with one of them (Vela *et al.*, 2000) recognizing 13 different biotypes based on acidification of tagatose, sucrose, mannitol and cyclodextrin, and the presence of the enzymes pyroglutamic acid arylamidase and N-acetyl-b-glucosaminidase. However, only 6 of these 13 biotypes were isolated from fish (Vela *et al.*, 2000). Ravelo *et al.* (2001) phenotypically characterized (by conventional and miniaturized systems) 23 strains of *L. garvieae* isolated from different fish species and geographic regions, having shown a high level of biochemical homogeneity among strains. Consequently, at least for fish, different biotypes should not be established, since they do not have epidemiological or intra-species taxonomic value (Ravelo *et al.*, 2001).

Molecular methods such as Random Amplified Polymorphic DNA (RAPD) (Colorni *et al.*, 2003; Ravelo *et al.*, 2003; Pereira *et al.*, 2004; Foschino *et al.*, 2008; Altun *et al.*, 2013), Repetitive Extragenic Palindromic (REP) DNA (Schmidtke & Carson, 2003), BOX-PCR (Schmidtke & Carson, 2003), Pulsed-Field Gel Electrophoresis (PFGE) (Vela *et al.*, 2000; Kawanishi *et al.*, 2005; Kawanishi *et al.*, 2006; Tejedor *et al.*, 2011; Tsai *et al.*, 2012), biased sinusoidal field gel electrophoresis (Madinabeitia *et al.*, 2009), *Sau*-Polymerase Chain Reaction (*Sau*-PCR) (Foschino *et al.*, 2008), Amplified Fragment Length Polymorphism (AFLP) (Foschino *et al.*, 2008), Restriction Fragment Length Polymorphism (RFLP) ribotyping (Eldar *et al.*, 1999; Eyngor *et al.*, 2004), DNA:DNA hybridization (Eldar *et al.*, 1999), and 16S rDNA sequencing (Chen *et al.*, 2002; Altun *et al.*, 2004; Kawanishi *et al.*, 2007; Sharifiyazdi *et al.*, 2010), have been used to study the molecular variability of *L. garvieae* isolated from fish species from all over the world. Some of these techniques (RAPD, PFGE and ribotyping) have also been used with the aim of resolving the epidemiological puzzle of how the disease has spread throughout European countries and around the world.

Ravelo *et al.* (2003) used RAPD to analyze a collection of 52 isolates of European and Japanese strains of *L. garvieae* isolated from rainbow trout, catfish and yellowtail, using two different oligonucleotide primers [P5 and P6 from the Ready-To-Go RAPD Analysis Beads (GE Healthcare)]. These authors verified that the patterns obtained allowed the differentiation of three genogroups, related to both the geographical

origin and the primary host of the isolates. Although strains showed minor pattern differences among them, the Spanish, Portuguese, Turkish and English trout isolates and a catfish strain from Italy clustered together in a close genetic relationship. Another genetic group was formed by French and Italian trout isolates, and finally the Japanese isolates from yellowtail constituted the third genetic group showing a great similarity with the *L. garvieae* reference strain NCDO 2155. In agreement with this, Foschino *et al.* (2008) observed that fish isolates from Spain, Italy and England clustered together when submitted to RAPD analysis, using two different oligonucleotide primers (P5 and M13). Also, in Portugal, when strains of *L. garvieae* isolated from different trout farms were analyzed by RAPD using primer P5, all strains were included within the genetic Group A of Ravelo *et al.* (2003) (Pereira *et al.*, 2004).

The presence of the same RAPD pattern in different countries could be easily explained by the movement of infected fish, contaminated eggs or asymptomatic carriers. On the other hand the presence of different RAPD patterns in the same country may be explained by the selective pressure induced by vaccination: the historical use of vaccines or the use of a distinct vaccine formulation.

All these results indicate the potential use of RAPD in epidemiological studies of *L. garvieae*.

In 2000, Vela *et al.* applied the PFGE technique to 84 strains of *L. garvieae* isolated from trout, cow, buffalo, human and water sources. When considering only the trout isolates, we can verify the existence of three genetically unrelated clones, one comprising Spanish and Portuguese isolates and the other two composed of the Italian and French strains respectively. Kawanishi *et al.* (2005) applied the PFGE technique to Japanese strains of *L. garvieae* isolated from fish and verified that different strains of *L. garvieae* isolated from the genus *Seriola* from a wide area in Japan have homogeneity in their PFGE patterns. This result suggests that isolates with the same origin have spread and caused lactococcosis in genus *Seriola* for 28 years in Japan. Also Tejadador *et al.* (2011) found that Spanish trout isolates generated only two pulsotypes, showing that the fish isolates were genetically homogenous, and concluded that outbreaks of *L. garvieae* in Spanish trout farms during the last decade belong to a single epidemic strain. This kind of result was also obtained by Tsai *et al.* (2012), who found that, independently of the fish species, all isolates of *L. garviae* in Taiwan showed a high level of genetic homogeneity, since only one pulsotype was obtained.

All these results confirm the idea that in those geographical areas or countries such as Japan, Spain and Twain where lactococcosis is endemic, the population of *L. garvieae* shows a clonal structure, whereas bacterial diversity characterizes sites where the infection is sporadic. Although many of these studies were carried out with different strains of *L. garvieae*, making the comparison of results difficult, they all point to the existence of at least three major clonal lineages in fish isolates of this bacterium. These lineages are associated with their geographical origin. These same studies also

point to the existence of genetic heterogeneity within *L. garvieae*, and showed the usefulness of RAPD, PFGE and ribotyping as epidemiological tools for *L. garvieae*.

Regardless of the inclusion in some works of Portuguese isolates of *L. garvieae* (Doménech *et al.*, 1993; Ravelo *et al.*, 2003), the genetic variability of this bacterium and the epidemiology of lactococcosis in Portugal was investigated only once (Pereira *et al.*, 2004). The present study investigates the biochemical and genetic relationship of different *L. garvieae* strains obtained from Portuguese trout farms from 1999 to 2009. To our knowledge it is also the first study worldwide where several different molecular techniques (RAPD, REP, BOX and PFGE) are employed at the same time to characterize strains of *L. garvieae*.

Streptococcus parauberis

Turbot, *Schophthalmus maximus* (Linnaeus 1758), is a very important farmed marine fish species in Europe. From 2000 to 2010 the average turbot production in Europe was 6.800 tonnes per year with the main producer countries being located in Southwestern Europe (FAO, 2010). During this time, the Portuguese production grew from 351 tonnes in 2008 to 3200 tonnes in 2012 (Estatística & Direcção-Geral de Recursos Naturais, 2012).

Streptococcal infections are caused by a variety of phenotypes belonging to several genera of Gram-positive bacteria, such as *Streptococcus*, *Lactococcus* (Eldar & Ghittino, 1999) and *Enterococcus* (Kusuda & Salati, 1993). Streptococcal infections are among the most important fish diseases caused by Gram-positive bacteria, and have been reported worldwide (Aoki *et al.*, 1990; Toranzo *et al.*, 1994; Eldar *et al.*, 1995; Eldar & Ghittino, 1999; Baeck *et al.*, 2006; Shin *et al.*, 2006; Ramos *et al.*, 2012) in wild and farmed populations of diverse freshwater and marine fish (Kusuda & Salati, 1993; Austin & Austin, 2012). The occurrence of streptococcal infections increased with intensification of culture practices (Eldar & Ghittino, 1999).

The first diagnosed outbreak of streptococcosis was in 1957 in rainbow trout cultured in Japan (Hoshima *et al.*, 1958). After that, several other important cultured fish species have become affected with this disease (Inglis *et al.*, 1993; Kusuda & Salati, 1993; Toranzo *et al.*, 1994; Doménech *et al.*, 1996; Alcaide *et al.*, 2000; Baeck *et al.*, 2006; Kim *et al.*, 2006; Austin & Austin, 2012). In 1993, an important epizootic outbreak of streptococcosis occurred in turbot cultured in Spain (Toranzo *et al.*, 1994). Although initially thought to be caused by an *Enterococcus* species-like bacterium, Doménech *et al.* (1996), using 16S ribosomal DNA (rDNA) analysis concluded that these fish isolates belonged to the species *Streptococcus parauberis* (Williams & Collins, 1990), formerly classified as *S. uberis* type II, a recognized pathogen of mammals (Doménech *et al.*, 1996).

S. parauberis infection in fish is a septicemic disease that occurs primarily during the summer months (Romalde & Toranzo, 1999), but is able to induce a chronic state with a low daily mortality rate occurring over several weeks (Roy & Ruth, 2002). The main clinical signs in affected fish are pronounced uni- or bilateral exophthalmia with an accumulation of mucopurulent exudate in the eyeball, and frequently in the dorsal region or at the base of the pectoral fins (Toranzo *et al.*, 1994; Ramos *et al.*, 2012). Sometimes, ascitic fluid can be observed in the peritoneal cavity (Doménech *et al.*, 1996; Ramos *et al.*, 2012).

Gram-positive cocci can be isolated on general purpose media but growth is enhanced on blood agar. Biochemical characterization can be accomplished by traditional tube and plate procedures as well as using commercial miniaturized systems (Toranzo *et al.*, 1994; Eldar & Ghittino, 1999; Ramos *et al.*, 2012).

No intraspecific classification of *S. parauberis* strains isolated from diseased turbot based on phenotypic characterization is possible, since they have shown a total homogeneity among strains (Toranzo *et al.*, 1994; Toranzo *et al.*, 1995a; Ramos *et al.*, 2012). However, in 2009 two different serotypes were proposed for *S. parauberis* strains isolated from Japanese flounder (Baeck *et al.*, 2006; Kanai *et al.*, 2009; Han *et al.*, 2011). Also, some molecular techniques like analysis of cell envelope proteins, did not allow the discrimination among isolates of this bacterial fish pathogen (Toranzo *et al.*, 1995a). For epidemiological studies and identification of isolates it is very important to have reliable methods for strain differentiation. Discriminative methods based on genotypic differences are not affected by the physiological state of the organism and can be easily standardized. Based on these premises, Romalde *et al.* (1999) investigated the intra- and interspecific relationship of *S. parauberis* using RAPD. These authors were able to observe genetic variability within this fish pathogen, and concluded that this technique is useful for discriminating between turbot isolates (Romalde *et al.*, 1999). Since then, no other investigations using other molecular techniques were performed in *S. parauberis*.

The control of this bacterium is achieved by vaccination with a formalin-killed bacterin (Toranzo *et al.*, 1995b; Romalde *et al.*, 1996b) or by chemotherapy. Nevertheless chemotherapy does not always work *in vivo* even though this bacterium can be sensitive to a variety of drugs *in vitro* (Romalde *et al.*, 1999; Ramos *et al.*, 2012). Streptococcosis seems to be endemic in some turbot farms, posing a putative danger of new outbreaks (Currás *et al.*, 2002; Ramos *et al.*, 2012).

In Portugal, tetracyclines are one of the main antibiotics used for the control of bacterial infections in fish. However, there is a high frequency of tetracycline-resistant bacteria among various fish pathogens and aquaculture environments (Aoki & Takahashi, 1987; Kusuda & Salati, 1993; Sano, 1998; Nonaka & Suzuki, 2002; Kim *et al.*, 2004; Maki *et al.*, 2008), with approximately 40 different tetracycline resistance determinants described (Roberts *et al.*, 2012). In streptococci the common tetracycline

resistance determinants are related to Gram-positive species *tet* genes, such as *tet*(K), *tet*(L), *tet*(M), *tet*(O) and *tet*(S) (Chopra & Roberts, 2001).

During the years of 2004 and 2005, outbreaks occurred in a turbot farm in the north of Portugal causing high mortalities and severe economic losses. The survey conducted during these outbreaks revealed the presence *S. parauberis*. The biochemical and molecular characterization of the *S. parauberis* isolated during these outbreaks will be reported in this study, as well as the search for tetracycline resistance genes. To our knowledge, there is only one previous report of streptococcosis in Portugal (Ramos, 2012), with this study being the first where the strains are genotyped using different molecular techniques.

Application of bacteriophages in aquaculture

Aquaculture is considered to be the fastest growing food production industry, boosted by governmental impulse and technological development. Consequently this industry is reducing the world's dependence on fisheries and offering financial relief to developing countries, which are the main contributors to this tremendous increase (Goldburg & Naylor, 2005). Portugal has not been an exception, particularly after its integration in the European Union (Gouveia, 1990; Gouveia, 1994).

However, in intensive aquaculture, the appearance of infectious diseases, especially those of bacterial etiology, are a major concern causing serious economic losses.

Ever since penicillin, the first antibiotic, was discovered 75 years ago, the treatment of bacterial infections has always relied on the use of these substances. However antibiotics have become increasingly ineffective (Teuber, 2001; Heuer *et al.*, 2006) due to widespread clinical, veterinary, and animal agricultural usage. We are now facing the threat of “superbugs”, i.e. pathogenic bacteria resistant to most or all available antibiotics (Parisien *et al.*, 2008). The World Health Organization has already warned that the world is heading back to a pre-antibiotic era due to the increase of multiple antibiotic-resistant pathogens. Additionally, antibiotics were found to be useful as growth enhancers in animal production. Unsurprisingly, since this discovery massive amounts of these substances have been used around the world. However molecular evidence indicates that industrial-scale use of antibiotics for the last 50 years has not only increased the diversity of antibiotic resistant pathogens but also has led to the spread of the most resistant strains (Levin, 1995; Baquero & Blázquez, 1997).

In aquaculture the control of bacterial diseases has always relied on antibiotics, which are often inadequately used by fish farmers. As this industry expands, questions arise concerning the consequences of the use of these substances, since these

drugs are administered by mixing them with feed that is dispersed in the water. Consequently, fish farms are directly dosing the environment, resulting in selective pressures in the exposed ecosystem (Angulo, 2000). Also, the use of antibiotics in fisheries is a highly controversial issue due to the possibility of tissue deposition, since aquaculture products with antibiotic residues can change the human intestinal microbiota and promote the appearance of allergies and toxicity problems (Cabello, 2006); the acquisition, by human pathogenic bacteria, of determinants of resistance to antibiotics developed in aquaculture systems (Alderman & Hastings, 1998; Ho *et al.*, 2000); environmental contamination from the use of large quantities of antibiotics in aquaculture might negatively affect the natural bacterial communities. Furthermore, antibiotic persistence in sediments (Hektoen *et al.*, 1995) may negatively affect the natural bacterial community and consequently the biogeochemical cycles (Fuhrman, 1999). Furthermore, the development of new antibiotics is not as profitable as the development of other drugs (e.g. those for treating chronic diseases, which are needed for long periods, instead of antibiotics, that are taken only for treating acute illness). For this reason, large pharmaceutical companies are not investing much in the development of new antibiotics, and consequently the appearance and spread of resistance among bacteria has been faster than the development of new antibiotics for combating them.

Several studies have assessed the impact of antimicrobial agents used in aquaculture on nonpathogenic bacteria found on fish farms and in their sediments. For instance, bacteria resistant to antimicrobial agents used on fish farms were isolated from sediment beneath the “netpens” of those farms (Björklund *et al.*, 1991; Coyne *et al.*, 1994; Kerry *et al.*, 1994). In other studies, resistant bacteria were isolated from natural and commercial fish species captured on fish farms (Ervik *et al.*, 1994; Kerry *et al.*, 1994), however, no resistant bacteria were found in fish from untreated areas (Ervik *et al.*, 1994).

The application of chemotherapy in aquaculture is not always successful since antibiotics are administered in the feed, and diseased animals show poor feeding habits (Morrison & Rainnie, 2004)

Fish pathogens that may have acquired resistance as a result of drug usage and the continuous presence of residual levels in fish can act as a host for resistance genes. The emergence of antimicrobial resistance following the use of antibiotics in aquaculture has been identified both in bacteria that are fish pathogens and those that are not (Alderman & Hastings, 1998; Angulo, 2000; Ho *et al.*, 2000).

Many resistance determinants in fish pathogens are carried on transferable R plasmids (Watanabe *et al.*, 1971; Aoki, 1988; Inglis *et al.*, 1993; Kruse & Sørum, 1994). Consequently, these resistance genes can be horizontally spread to other bacteria, including human pathogens (Aoki, 1997), and this has been demonstrated in bacteria from fish ponds (Aoki, 1997) and in marine sediments (Stewart & Sinigalliano, 1990). *In vitro* and in simulated natural microenvironments, plasmids carrying resistance

determinants have been transferred from fish pathogens to human and animal pathogens (Hayashi *et al.*, 1982; Nakajima *et al.*, 1983; Sandaa *et al.*, 1992; Kruse & Sørum, 1994; Son *et al.*, 1997).

In 1991 a new cholera epidemic began in Ecuador and it was demonstrated that the original epidemic strain of *V. cholerae* O1, that was susceptible to the 12 antimicrobial agents, became resistant due to the transference of multidrug determinants from *Vibrio harveyi* (a shrimp pathogen) to *V. cholera* (Weber *et al.*, 1994)

Due to all these concerns, the use of antibiotics in aquaculture has been greatly limited or banned in most countries. Consequently, new solutions for the treatment of bacterial fish pathogens are urgently needed. Several antimicrobial approaches for the treatment of bacterial infections in aquaculture have been proposed: probiotics (Gibson *et al.*, 1998; Moriarty, 1998; Verschuere *et al.*, 2000; Nikoskelainen *et al.*, 2003; Farzanfar, 2006) immuno-stimulants (Vadstein, 1997; Bricknell & Dalmo, 2005), bacteriocins (Riley & Wertz, 2002; Shehane & Sizemore, 2002), polyculture (Tendencia, 2007), disruption of bacterial quorum sensing (Defoirdt *et al.*, 2004; Defoirdt *et al.*, 2007; Bai *et al.*, 2008) and phage therapy (Nakai & Park, 2002), among others (Defoirdt *et al.*, 2011).

One of the proposed solutions is phage therapy. Phage therapy is slowly making its way back into research after a hiatus of over forty years. Phages are the most abundant life form on Earth and can be found virtually anywhere (Nation, 2003; Kutter & Sulakvelidze, 2005), occupying all habitats of the world where bacteria thrive. They are the most abundant and the most versatile group of organisms on Earth; in environmental samples phages usually outnumber their bacterial hosts 10 to 100-fold (sometimes up to 1000-fold), and the global phage population has been estimated to approach 10^{31} (Wommack & Colwell, 2000; Weinbauer, 2004; Brüssow & Kutter, 2005; Skurnik *et al.*, 2007).

Phages are naturally-occurring viruses, composed of nucleic acids (dsDNA, ssDNA or ssRNA) encapsulated in a protein coat, which infect prokaryotes as part of their natural life cycle, being incapable of attacking other cells or organisms. Phages are viruses that infect bacterial hosts and depend on bacterial processes to replicate themselves. Upon infection, phages either actively produce progeny, which are assembled within the cytoplasm, or can integrate into the bacterial chromosome (Goodridge & Abedon, 2003). Progeny are released into the extracellular environment either through lysis (using lytic enzymes to destroy the cell wall) or via extrusion whereby filamentous virions move out of the cell without causing bacterial death (Abedon *et al.*, 2001). Lytic phages are the most predominant and given their virulent nature against the prokaryotic host, are the most amenable to therapeutic applications.

When compared to antibiotics, phages are superior to these substances since they can be effective against multidrug-resistant pathogenic bacteria. The appearance of phage-resistant mutants is not as common as that of antibiotic resistance, and it

is relatively easy to isolate phages capable of infecting the mutated bacterial strain. Furthermore, if the phage receptor on the bacteria functions as a virulence determinant (such as lipopolysaccharide), it can be assumed that a mutation eliminating the receptor would attenuate the bacterium, making it easier for the host immune system to eliminate the bacteria (Skurnik *et al.*, 2007). In addition, phage-associated side effects are uncommon since phages or their products do not affect eukaryotic cells; being only composed of proteins and nucleic acids, their breakdown products consist exclusively of amino acids and nucleic acids. Thus, they are not xenobiotics and, unlike antibiotics and antiseptic agents, their introduction into a given environment may be seen as a natural process.

Phages display remarkable host specificity and are incapable of attacking other cells or organisms. Their specificity for a particular bacterial species or strain reduces the negative impact associated with the use of antibiotics such as development of resistance, damage to the environmental bacterial community or to intestinal microflora. In the presence of susceptible bacteria, phages rapidly increase in number, which results in a more effective treatment. Phages delivered *in vivo* can thus theoretically access infected tissues by blood stream circulation, and in the presence of the susceptible pathogenic bacteria go deeper into problem loci of infection where antibiotics often cannot penetrate in sufficient concentration to be useful. Moreover the self-replicating nature of phages makes the need for multiple administrations unnecessary (Dubos *et al.*, 1943; Smith & Huggins, 1982; Barrow & Soothill, 1997; Kutter & Sulakvelidze, 2005). The use of bacteriophages as agents for the control of bacterial infections in aquaculture has advantages and drawbacks (Table 1).

The use of phages to prevent and treat bacterial fish diseases was suggested for the first time in 2002 (Nakai & Park, 2002). Since it is a relatively new research field there are only a few published studies, and this is particularly true for the treatment of bacterial fish pathogens. Japan (Park *et al.*, 1997; Nakai *et al.*, 1999; Park *et al.*, 2000; Park & Nakai, 2003) and the United States (Nation, 2003; Stannard *et al.*, 2005) are the only countries where experimental work has been done using phages to treat naturally occurring bacterial fish infections.

A number of phages have been isolated for potential use in phage therapy against important fish and shellfish pathogens, such as *Aeromonas salmonicida* ssp. *salmonicida* (Paterson *et al.*, 1969; Popoff, 1971a; Popoff, 1971b; Rodgers *et al.*, 1981; Nation, 2003; Imbeault *et al.*, 2006; Verner–Jeffreys *et al.*, 2007), *Flavobacterium psychrophilum* (Stenholm *et al.*, 2008; Kim *et al.*, 2010; Laanto *et al.*, 2011), *Flavobacterium columnare* (Prasad *et al.*, 2011), *Vibrio harveyi* (Munro *et al.*, 2003; Karunasagar *et al.*, 2005; Pasharawipas *et al.*, 2005; Vinod *et al.*, 2006; Karunasagar *et al.*, 2007; Okano *et al.*, 2007; Shivu *et al.*, 2007; Crothers-Stomps *et al.*, 2010; Phumkhachorn & Rattanachaikunsopon, 2010), *Pseudomonas plecoglossicida* (Park *et al.*, 2000; Park & Nakai, 2003), *Edwardsiella tarda* (Wu & Chao, 1982; Wu *et*

Table 1 - Pros and cons of bacteriophage therapy (adapted from Oliveira *et al.*, 2012 and Sandeep, 2006).

Issue	Advantage	Disadvantage
Abundance	Ubiquitous, providing a large, naturally available pool of bacteriophages	Strongly lytic phages have to be selected from the available pool
Multiplication/Self limitation	Exponential replication in the presence of host bacteria, and rapid decline in its absence. Repeated administration is not necessary	<i>In vitro</i> growth data is difficult to extrapolate to <i>in vivo</i> . <i>In vivo</i> data is difficult to interpret and to generalize from one situation to another
Host specificity	Narrow host range, avoids the infection of other bacteria, namely useful bacteria and the normal intestinal microflora Reduced possibility for the development of secondary infections, and side effects are less likely to occur	The exact host bacterium causing the infection needs to be identified Strain specific rather than species specific, increasing the difficulty when preparing phages for highly diverse bacterial variants
Bacterial debris	Removal can be readily achieved by current technology	Might cause therapy to fail since it may be fatal for the injected organism
Administration	Through impregnated feed, injection or by immersion	Poor feeding habits of diseased animals Injection might be impractical for the treatment of large numbers of animals
Dose	Determination of the precise initial dose may not be essential since phage titers increase along with bacterial infection	Limited data available on effective phage doses
Fate	Phage titer decreases after killing the target bacteria Phages are easily excreted out of the animal body and do not pose any environmental risk	The administered dose must account for the phages that are quickly excreted
Multiple infection	A cocktail of phages, each specific to the target bacteria can be applied	All the infecting bacteria must be previously identified
Bacterial resistance	Overcoming resistance should not be difficult due to the worldwide availability and rapid mutation of phages Phage-resistant bacteria could become virulent, since the development of resistance could be accompanied with the loss of the virulence determinants	Phage-resistant mutants are fairly common and develop rapidly Newly isolated phages require efficiency tests

al., 1983; Matsuoka & Nakai, 2004), *Edwardsiella ictaluri* (Walakira *et al.*, 2008), *Lactococcus garvieae* (Park *et al.*, 1997; Park *et al.*, 1998; Nakai *et al.*, 1999), *Streptococcus inae* (Matsuoka *et al.*, 2007), *Vibrio anguillarum* (Wu & Chao, 1984), *Aeromonas hydrophila* (Wu *et al.*, 1981; Chow & Rouf, 1983; Merino *et al.*, 1990; Stannard *et al.*, 2005) and *Yersinia ruckeri* (Stevenson & Airdrie, 1984). Most of these studies demonstrated the potential use of phages to reduce the impact of bacterial infections, with a positive effect on fish survival. In Japan, studies showed that fish injected with phages against *L. garvieae* had a 45 % greater survival rate (Nakai *et al.*, 1999). These studies demonstrated that phage therapy has great potential in protecting fish experimentally infected with pathogenic bacteria (Park *et al.*, 1997; Park *et al.*, 1998;

Nakai *et al.*, 1999; Nakai & Park, 2002; Park & Nakai, 2003). In Taiwan it has been shown that when loaches (*Misgurnus anguillicaudatus*) were submerged for 8 hours in an infected solution of *E. tarda* (at a concentration 1×10^8 CFU/ml), and with the presence of a phage against this bacterium at an MOI (Multiplicity Of Infection) of 0.1, 90 % of the fish survived for more than 4 days. In the control group (only infected with the bacteria) more than 70 % of the fish died (Wu & Chao, 1982). These same authors were able to avoid outbreaks of *V. anguillarum* in a fish farm, using phages against this bacterial species (Wu & Chao, 1984; Wu *et al.*, 1986). However, one study shows that furunculosis in Atlantic salmon is not readily controllable by application of bacteriophages (Verner–Jeffreys *et al.*, 2007). Phage therapy has been explored as a stand-alone treatment or a way to supplement antibiotic treatments. Due to these contradictory results the use of phages as alternative to antibiotics for the treatment of bacterial fish pathogens needs to be further researched (Stenholm *et al.*, 2008).

These contradictory results could be related to the many and sometimes difficult steps necessary to establish a successful phage treatment. If a phage collection already exists and has been studied, the first step in phage therapy is the establishment of the causative agent of the disease. Afterwards, bacteriophages must be selected that can effectively infect the target bacteria and evaluate their potential to control the bacterial infection in an aquaculture setup. However, if no phage collection exists, more steps are involved and have to be done sequentially: (1) isolation of bacteriophages from the environment, using an enrichment method; (2) culture of bacteriophages; (3) bacteriophage typing of the target bacterium; (4) selection of lytic bacteriophages for therapeutic use; (5) phenotypic and genotypic characterization of the bacteriophages; (6) recognition of the existence of virulence genes or other toxic factors in the phage nucleic acid; (7) assessment of therapeutic efficacy of phages against experimental infections and natural infections.

Phage efficacy in experimental and natural infections has to be established before phage therapy, since phages that show a strictly lytic cycle in a well defined *in vitro* environment, may not remain lytic under normal physiological conditions found in a body. For example in certain circumstances the phage may change to adapt to a lysogenic cycle (Sandeep, 2006). Phage efficiency evaluation trials in an aquaculture setup are also useful for establishing the infection dose and route of phage administration. Contrary to antimicrobials and other chemicals, the precise determination of the initial dose may not be essential, due to the self-replicating nature of phages; phage titers will increase along with the bacteria concentration in infected individuals or in pathogen-contaminated water (Nakai, 2010).

In the last 15 years, phage therapy has seen a revival, being the subject of intensive research in many fields. However, the application of phage therapy for the treatment of bacterial infections in fish is not yet fully investigated, and both successful and unsuccessful results have been observed. Before being deployed extensively the

impact of this biocontrol technique in aquaculture systems, fish and humans must be taken into account. Also, the development of bacterial resistance should not be neglected, even though the selective pressure for the development of resistance seems more unlikely in bacteriophages than in conventional antimicrobials. The cost and delivery route must be considered and studied, such as the administration of phage cocktails combined with antimicrobial therapy. One of the main hurdles that the use of phage therapy in an aquaculture setup will face is approval by regulatory authorities. However, the fact that phage sprays are already approved for use in food items, may facilitate this approval.

The application of phage therapy in aquaculture seems to have some advantages mainly related to the fact that the infection route is the same as the medium in which the animals live, and also the versatility of administration routes and the possibility of use in closed or open systems. Consequently, the potential for naturally occurring bacteriophages to be used in therapy or prophylaxis of bacterial diseases in aquaculture is a wide and promising field.

Objectives

In 2010 as part of a project aimed at intensifying the production of sole (*Solea senegalensis*) by A. Coelho & Castro (ACC) a microbiological study was undertaken on their facilities. The objectives of this survey were:

1. Determine the water microbial load at representative places in the facility in order to evaluate the disinfection efficiency of different filters;
2. Study the distribution of *Tenacibaculum maritimum*, the only fish pathogen already known to be present, both through isolation on bacteriological media, and PCR detection from fish, fish mucus, water, sediment and biofilm from representative places in the facility;
3. Using the same methodology, search for the presence of other important bacterial fish pathogens, namely *Vibrio anguillarum*, *Photobacterium damsela* ssp. *piscicida*, *Streptococcus parauberis* and *Lactococcus garvieae*.

Very few studies related to the characterization of bacterial fish pathogens exist in Portugal. Until now, only three researchers (Pereira *et al*, 2004; Marques, 2010; Mendes, 2010) studied by molecular techniques the characteristics of the Portuguese bacterial fish pathogens. Since 1998 our lab has given, more or less continuously, support to fish farmers. During this time, a collection of different bacterial fish pathogens was assembled, and three of these species (*Aeromonas salmonicida* ssp. *salmonicida*, *Lactococcus garvieae* and *Streptococcus parauberis*) are characterized in this work.

Also during this time we witnessed the development of antimicrobial resistance to several antimicrobial agents following their use in the farms, leading to the realization that new solutions for treatment of bacterial fish pathogens were needed. The venue that was pursued was phage therapy.

Taking into account these observations the aims of this study were:

- 1) Give an overview of the Portuguese aquaculture bacterial infections:
 - Study the biochemical and molecular characteristics of *Aeromonas salmonicida* ssp. *salmonicida*, *Lactococcus garvieae* and *Streptococcus parauberis* strains isolated in Portugal;
 - Determine the usefulness of the molecular typing methods as tools for the epidemiology of these bacteria.
- 2) Isolate phages against several bacterial fish pathogens:
 - Study the biological and molecular characteristics of the isolated phages;

Material & Methods

1. Sample Collection

1.1. Water Collection

For water analysis, samples were collected monthly during Autumn, Winter and Spring, and weekly during Summer at ACC's main water intake from the sea (P1, Fig. 1) and at different sampling points representative of the whole circuit in the facility (Fig. 1). All water coming directly from the sea passed through a sand (P2) and an UV filter (P3), before being sent directly to the adult tanks. Water to the hatchery and nursery additionally passed through a skimmer (P4), a cartridge filter system, another UV system (P5) and a degasser, before entering the hatchery tanks (P6). P7 was set after the nursery tanks. All water coming from the facility was collected in a sedimentation tank (P8). Part of this water passed through a skimmer with ozone (P9) and recirculated back into the adult tanks. At the end of July 2009, P4 was eliminated, since the skimmer was removed from the circuit.

Water samples were collected in 1 l sterile glass bottles. Samples of sediment and the biofilm that accumulated around the discharging pipe of a number of tanks in the facility were collected in 100 ml sterile plastic bottles. All samples were immediately transported to the laboratory in refrigerated containers and processed within one hour of collection. Upon arrival each water sample was serially diluted (up to 10^{-7}) and 100 μ l of each dilution were inoculated in duplicate onto plates of Marine Agar (MA; Pronadisa, Spain), Tryptic Soy Agar (Merck, Germany) supplemented with 1 % (w/v) NaCl (TSA-1), Thiosulfate-Citrate Bile Sucrose (TCBS; Merck, Germany), *Flexibacter maritimus* Medium [FMM; (Pazos *et al.*, 1996)] and Columbia Agar with 5 % sheep blood (BA; bioMérieux, France). MA plates were used for the determination of the bacterial load in the water (colony forming units - CFU/ml), whereas plates of other media were used for the isolation of fish pathogenic bacteria.

For bacterial counts, plates of MA with 30-300 colonies were selected and results registered as the number of CFU/ml of water. In this medium the most representative types of colonies were selected and re-streaked until purity was obtained. In all other media, colonies with morphological characteristics consistent with the searched pathogenic bacteria were selected and re-streaked until purity was obtained. All pure bacterial cultures were subjected to some basic physiological and biochemical tests.

1.2 - Bacterial isolation

1.2.1 – Bacterial isolation from sole

On each sampling date, 10 to 15 sole were taken to the laboratory for bacteriological analysis and mucus collection. In total 312 sole with signs of disease were analysed. Although turbot and European sea bass were also reared at the facility, these species never showed any signs of disease, and consequently none were analysed for the

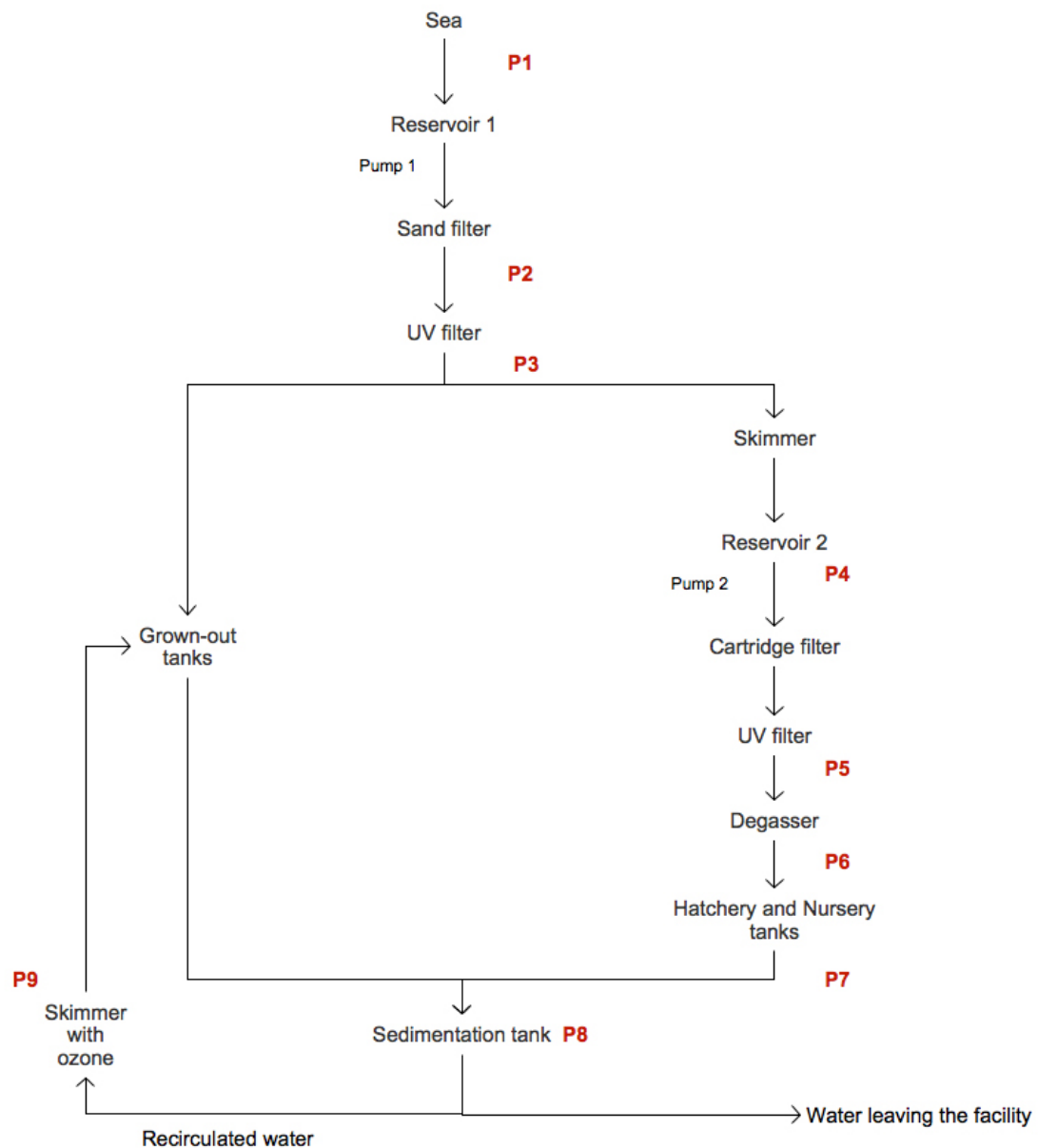


Figure 1 - Schematic representation of the water flow in the fish farm, location of the filters and sampling points (P1-P9) at the beginning of the study.

presence of bacteria. Disease signs in sole included mouth erosion and/or lesions in the trunk, which were very often ulcerated. Samples of internal organs (kidney, liver and spleen) were streaked onto plates of TSA-1, MA, FMM, BA and TCBS. Whenever an ulcerated skin lesion was observed, a sterile loop was used to touch its advancing edge and streak onto FMM, in an attempt to isolate *Tenacibaculum maritimum*. All plates were incubated at 25 °C for 24-36 h, except FMM plates, which were incubated at 20 °C for 72 h.

Other than MA, all bacteriological media used were intended to allow the isolation of pathogenic bacteria, from both water and fish tissues.

All TSA-1 and BA plates were scrutinized for the presence of bacterial colonies

with characteristics consistent with *Vibrio anguillarum*, *Photobacterium damsela* ssp. *piscicida* and *Streptococcaceae* (*Streptococcus parauberis* and *Lactococcus garvieae*), as described by Austin & Austin (2012). Plates with TCBS and FMM were thoroughly observed for detection of *V. anguillarum* and *T. maritimum*, respectively, as described by Austin & Austin (2012) and Pazos *et al.* (1993). All colonies suspected of belonging to these bacterial species were purified in the same detection medium and subjected to some biochemical and physiological tests (Gram, oxidation/fermentation of glucose, shape, mobility, presence or absence of oxidase and catalase and amino acid decarboxylation). Whenever the phenotypical characteristics of the colonies and the results of these tests were consistent with one of the above fish pathogens, the respective PCR protocol was followed for identification.

1.2.2 - *Aeromonas salmonicida* ssp. *salmonicida*

From April 1998 to July 1999, high mortalities were observed in regular visits to two trout (*Oncorhynchus mykiss*, Walbaum, 1792) farms located in the north of Portugal. These farms belong to different hydrographic basins and were located far from one another; one was located on a small river (strains IN) and the other on a dam (strains CA) (Table 4). However, during the sampling period a frequent transfer of animals from IN to CA was observed, where the final growth of the trout occurred.

In 2000 and 2001, during a research project, we visited a third trout farm, located on a dam in a different hydrographic basin from the other two. On this farm *Aeromonas salmonicida* ssp. *salmonicida* was isolated in several fish without external symptomatology (strains SRab) (Table 2).

Some of the animals from IN and CA showed melonosis, slight exophthalmia, blood-shot fins and lesions resembling boils, i.e. furuncles, in the musculature. Internally, haemorrhaging in the liver, swelling of the spleen, and multiple haemorrhages in the muscle and other tissues

Sterile swabs from liver, kidney, spleen and lesions (when present) were taken from all animals, streaked on TSA-1 and incubated at 22 °C for 24/48 h. Single colonies producing a brown pigment were re-streaked on TSA-1 to obtain pure isolates. A total of 43 isolates of *Aeromonas salmonicida* ssp. *salmonicida* were obtained during this sampling period (Table 2).

Table 2 - Place, date and number of isolates of *A. salmonicida* ssp. *salmonicida* used in this study and their origin.

Farm	Date of isolation	Number of Strains
CA	October, 1998	2
	November, 1998	2
	January, 1999	2
	February, 1999	2
	April, 1999	2
	May, 1999	3
	June, 1999	6
IN	April, 1998	1
	June, 1998	1
	July, 1998	1
	September, 1998	3
	October, 1998	4
	November, 1998	3
	December, 1998	1
	January, 1999	2
	March, 1999	2
	April, 1999	2
	May, 1999	2
	June, 1999	1
SRab	October, 2000	2
	March, 2001	1
Type Strain ATCC14174		1
Spanish isolates		2
American isolates		5

1.2.3 - *Lactococcus garvieae*

From June 2004 to October 2009, high mortalities were observed in visits to five different rainbow trout farms located in the north of Portugal. Diseased fish showed uni- or bilateral exophthalmia with abscesses and haemorrhages. Moribund specimens were collected and killed by spinal cord severance. All fish were subjected to necropsy in order to determine the etiology of the mortalities. A total of 66 fish (weight range 0.9 – 581.5g) were examined.

Sterile swabs from liver, kidney, spleen, brain, eyes, and if present, lesions, were streaked on BA and TSA-1. The inoculated plates were incubated at 25 °C for 48 h. Single colonies from plates with culture growth were re-streaked on TSA-1 to obtain pure isolates.

A total of 34 strains were obtained during this sampling period (Table 3).

1.2.4 - *Streptococcus parauberis*

From March 2004 to February 2005, during routine visits to a grow-out facility of turbot (*Scophthalmus maximus*, Linnaeus, 1758) located in the north of Portugal, high mortalities were observed. Diseased fish showed uni- or bilateral exophthalmia

Table 3 - Place, date and number of isolates of *Lactococcus garvieae* used in this study.

Fish Farm	Date of isolation	Strains analyzed
NFM	July, 2005	2
PC	October, 2004	7
	October, 2009	4
TPV	July, 2004	3
	August, 2009	9
	September, 2009	5
TG	June, 2004	2
TC	June, 2004	2
Spanish isolate (PP62.1)		1
Type Strain (NCDO 2155)		1

with abscesses, haemorrhages and accumulation of purulent fluid in the periorbital tissues. In some animals accumulation of purulent fluid at the base of the fins was also observed. In this facility fish were held in circular concrete tanks with an open flow circuit of seawater supplemented with oxygen. Monthly, moribund specimens were collected and killed by spinal cord severance and subjected to necropsy in order to determine the etiology of the high and progressive mortalities. A total of 110 fish (weight range 90 - 1020g) were examined.

Sterile swabs from liver, kidney, spleen, brain, eyes, and if present, lesions, were streaked on BA, TSA-1 and TCBS. The inoculated plates were incubated at 25 °C for 24-48 h. Single colonies from plates with culture growth were re-streaked on TSA-1 to obtain pure isolates.

A total of 26 strains were obtained during this sample period (Table 4).

All stock cultures were maintained at -80 °C in Tryptic Soy Broth (Merck, Germany) supplemented with 15 % (v/v) glycerol and 0.5 % (w/v) NaCl.

Table 4 - Date and organ of isolation of the *Streptococcus parauberis* strains used in this study.

Fish Farm	Date of isolation	Organ of isolation	Strains analyzed
CM	March, 2004	Brain	2
	June, 2004	Kidney	7
		Eye	
	June, 2004	Kidney	4
	August, 2004	Kidney	9
	September, 2004	Kidney	5
		Spleen	
	October, 2004	Kidney	2
		Spleen	
	January, 2005	Kidney	2
South Korean strains			2
Type strain NCDO 2020			1

2. Histological sampling and processing

Samples of liver, kidney, spleen, brain, eyes and skin from turbot were fixed in 10 % phosphate-buffered formalin, routinely processed, sectioned at 2 µm thick, stained with haematoxylin and eosin (H&E), Gram, Gram-Twort and Periodic Acid Schiff's (PAS), and examined under an optical microscope.

3. Biochemical and physiological characterization of the bacterial isolates

All isolated bacteria were subjected to morphological, physiological and biochemical standard plate and tube tests. Gram, shape, motility, presence or absence of oxidase and catalase, oxidation/fermentation of glucose and aminoacid decarboxylase were analysed. Gram reaction was performed according to Ryu (1938) and oxidase, catalase, morphology, motility, fermentation of glucose and aminoacid decarboxylation were performed according to Mac Faddin (1993) and Barrow & Gelthan (2003).

Based on the results from these tests bacteria isolated from water were further characterized biochemically (Mac Faddin, 1993; Barrow & Gelthan, 2003), and the isolates suspected to belong to a bacterial fish pathogen were subjected to confirmation by PCR.

All media used in physiological and biochemical identification of *S. parauberis* was supplemented with 1 % (w/v) NaCl. Acid production from carbohydrates was tested under aerobic and anaerobic conditions with ZOF medium (Lemos *et al.*, 1985) supplemented with 1 % of the appropriate sugar, and results were determined after 7 days of incubation.

Unless otherwise stated, strains were incubated at 22 °C or 25 °C for all tests. Tests were read daily during an incubation period of 2 weeks.

The ability of *L. garvieae* and *S. parauberis* to grow at 4, 10, 25 and 40 °C was tested in TSA-1 over a period of 2 weeks. Growth at 1, 4.5, and 6.5 % (w/v) NaCl was determined in TSA, supplemented with the correct percentage of NaCl at 25 °C, after 1 week of incubation.

In all tests reference strains of *A. salmonicida* ssp. *salmonicida* (ATCC 14174), *L. garvieae* (NCDO 2155) and *S. parauberis* (NCDO 2020) were included, along with 7 isolates of *A. salmonicida* ssp. *salmonicida* from Spain and the United States, one *L. garvieae* from Spain and two *S. parauberis* strains from South Korea (A270 and 554).

Biochemical identification was also performed with the commercial identification systems API 20E and API 20NE (BioMerieux, France) for *A. salmonicida* ssp.

salmonicida according to the manufacturers recommendations with the exception of the incubation temperature (22 °C). For the identification of numerical profiles API 20E Web V4.1 and API 20NE Web V7.0 were used.

L. garvieae and *S. parauberis* were also analysed with the commercial identification systems API 20Strep and RAPID 32Strep (BioMerieux, France) with an incubation temperature of 25 °C, and results were read at 4 and 24 h. Standardized inoculum was prepared from bacteria grown in blood agar plates for 18 h. Bacteria were suspended in tubes containing sterile saline solution and cell density was adjusted spectrophotometrically to OD = 0.8 (A580). For the identification of numerical profiles API 20Strep Web V7.0 and RAPID 32Strep Web V3.0 were used.

L. garvieae was also biochemically identified using a miniaturized system, according to the protocol of Carson *et al.* (2001).

4. Hemolysis Test

A test for hemolysis was conducted with pure isolates of *L. garvieae* and *S. parauberis* using BA at 25 °C for 24 h.

5. Antimicrobial susceptibility test

A. salmonicida ssp. *salmonicida* and *S. parauberis* antibacterial susceptibility was examined according to a standard method recommended by the National Committee for Clinical Laboratory Standards (2002), with Mueller-Hinton agar (Merck) and susceptibility test disks (Oxoid, Thermo Scientific, USA) that contained the antibiotic in test. The susceptibility of *A. salmonicida* ssp. *salmonicida* was tested against Oxolinic acid (OA-2), Furazolidon (FX-100), Chloramphenicol (C-30), Nalidixic Acid (NA-30), Oxytetracycline (T-30), Ampicillin (AM-10), Sulphonamide (G-25), Streptomycin (S-10), Tetracycline (TE-30). The susceptibility of *S. parauberis* was tested against Penicilin G (P-10), Erythromycin (E-15), Ampicillin (AM-10), Trimethoprim-Sulfametoxazol (SXT-25), Cefalotin (30), Chloramphenicol (C-30), Nitrofurantoin (F/M300), Furazolidon (500), Tetracycline (TE-30) and Streptomycin (S-10).

6. Serological identification

A. salmonicida ssp. *salmonicida* strains were serologically identified using the

BIONOR Mono-As kit which utilizes latex particles coated with specific polyvalent sheep antisera against *Aeromonas salmonicida* ssp. *salmonicida*. The specific antiserum binds to the microorganisms, obtained from a pure culture, resulting in an agglutination of the latex particles and thus providing the identity of the bacterial culture. The kit also contains a negative control reagent consisting of monodispersed latex particles coated with non-specific protein. The kit was used in accordance with the manufacturer's instructions. Positive results were recorded only if clear agglutination was seen within 30 s after mixing the test reagent with the bacteria and if no reaction was observed with the negative control reagent.

S. parauberis serological identification was performed by slide agglutination, mixing a sample of the bacteria with 10 µl of anti-*Streptococcus parauberis* serum. A negative control was made with deionized water. This serum was a kind offer from Dr. Alicia Estevez Toranzo from University of Santiago de Compostela (Spain).

7. Plasmid isolation

The presence of plasmids in *A. salmonicida* ssp. *salmonicida* was determined according to the method of Kado & Liu (1981) and Birnboim & Doly (1979) with the alterations proposed by Johnson & Woodford (1998). Samples were electrophoresed (25 V, 14 h) on a 0.7 % agarose gel in 0.5 % TBE Buffer. A 0,05 - 1 M basepair (bp) DNA mass marker (Bio-Rad, USA) was used. After electrophoresis, the gel was stained with 5 µg/ml of Ethidium Bromide for 30 min and destained for 30 min in distilled water. The gels were then visualized by UV trans-illuminator and photographed.

8. Extraction of bacterial DNA

DNA from water samples was extracted using the UltraClean Water Isolation Kit (MoBio Laboratories, Inc., USA); DNA from fish mucus, sediment and biofilm was extracted using the UltraClean Soil DNA Isolation Kit (MoBio Laboratories, Inc., USA), all according to the manufacturer's instructions. After extraction, all DNA samples were maintained at -20 °C until PCR analysis.

A. salmonicida ssp. *salmonicida* DNA was isolated using the E.Z.N.A® Bacterial DNA Kit (OMEGA Bio-tek, USA) following the manufacturer's recommendations. DNA from *L. garvieae* and *S. parauberis* was extracted with InstaGene Matrix (Bio-Rad) according to manufacturer's recommendations.

After extraction the concentration and purity of the DNAs were quantified spectrophotometrically (NanoDrop ND1000, Thermo Scientific), and their concentration

adjusted to 15 ng/μl. Purified DNA was maintained at -20 °C until use for PCR reactions. Unless otherwise stated, five microliters (75 ng) of each DNA solution was routinely used in the amplification reactions.

9. PCR Identification

PCR protocols used for pathogen identification were those developed by Gustafson *et al.* (1992) and Miyata *et al.* (1996) for *A. salmonicida* ssp. *salmonicida*, Avendaño-Herrera *et al.* (2004) for *T. maritimum*, Gonzalez *et al.* (2003) for *Vibrio anguillarum*, Osório *et al.* (2000) for *Photobacterium damsela* ssp. *piscicida*, Zlotkin *et al.* (1998) for *L. garvieae* and Hassan *et al.* (2001) for *S. parauberis* using the specific primers and conditions indicated by the respective authors.

T. maritimum was already known to be present in ACC and very often fish with signs consistent with infection by this bacterium were observed. In an attempt to evaluate the distribution of this pathogen throughout the facility, a PCR study was performed using DNA extracted from water, fish mucus, sediment and biofilm.

All PCR reactions were performed in a temperature-gradient thermocycler (Thermo Scientific), and the amplification was carried out with Supreme NZYTaQ 2x Green Master Mix (nzytech, Portugal) to which primers (10 μmol/μl), DNA template (75 ng) and ultrapure, sterile water were added to produce a final reaction volume of 25 μl. After amplification samples were kept at 4 °C until analyzed.

Unless otherwise stated, all samples were electrophoresed (90 V, 1 h) on a 1 % agarose gel stained with 5 μg/ml of SYBR Safe DNA gel stain (Invitrogen) in SB buffer (10 mM NaOH; 38mM Boric Acid). A 100-3000 basepair (bp) DNA mass marker (Solis-Biodyne, Estonia) was used as a molecular mass marker. After electrophoresis, gels were visualized by UV trans-illuminator and photographed.

10. Detection of antibiotic resistance genes

The presence of antimicrobial resistance genes in *S. parauberis* was investigated using either single or multiplex PCR assays. The oligonucleotide primer sets used to detect tetracycline resistance genes [*tet*(M), *tet*(O), *tet*(S), *tet*(M/O/S) and *tet*(K)] were derived from published sequences (Park *et al.*, 2009). Amplification of the *tet*(M), *tet*(O) and *tet*(S) genes was performed using multiplex PCR, as described by Ng *et al.* (2001). Amplification of the *tet*(M/O/S) and *tet*(K) genes was performed using a modification of the single PCR assay described by Sapkota *et al.* (2006). Using these primers and protocols a 406 bp PCR amplification product was expected for *tet*(M),

515 bp for *tet*(O), 667 bp for *tet*(S), 860 bp for *tet*(K), 267 bp for *tet*(L) and 685 bp for *tet*(M/O/S) respectively.

Amplification and electrophoresis was carried out as previously described. Electrophoresis fragments were subsequently visualized by UV trans-illuminator and photographed.

11. Molecular fingerprinting

All strains were typed by Random Amplification of Polymorphic DNA (RAPD) PCR, Repetitive Extragenic Palindromic (REP) PCR, and BOX-PCR. *A. salmonicida* ssp. *salmonicida* were also typed by Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR.

RAPD analysis was performed using the Ready-To-Go RAPD Analysis Beads (GE Healthcare, UK) for *A. salmonicida* ssp. *salmonicida* and *L. garvieae*. *L. garvieae* strains were also typed using the primer M13 [5'-GAGGGTGGCGGTTCT-3'; (Rossetti & Giraffa, 2005)], as were the *S. parauberis* strains. For *A. salmonicida* ssp. *salmonicida*, an initial study using all six distinct random 10-mer primers supplied in the kit and three bacterial isolates was performed. Afterwards, all bacterial isolates were analysed using primer 4 of the kit. Amplification and electrophoresis were carried out according to manufacturers instructions. *L. garvieae* strains were analysed using primer 5 following the recommendations of Ravelo *et al.* (2003). Amplification was carried out as follows: an initial denaturation step at 95 °C for 5 min; 30 cycles of 95 °C for 1 min (denaturation), 35 °C for 1 min (annealing) and 72 °C for 2 min (DNA chain extension). RAPD products were electrophoresed for 2 h on a 2 % agarose gel and processed as previously described.

REP-PCR was performed as described by Versalovic *et al.* (1991) and Versalovic *et al.* (1994) using primers REP1R-I (5'-IIICGICGICATCIGGC-3') and REP2-I (5'-IIICGNCGNCATCNGGC-3') for *A. salmonicida* ssp. *salmonicida*, and (GTG)₅ (5'-GTGGTGGTGGTGGTG-3') for *L. garvieae* and *S. parauberis*.

BOX-PCR was performed as described by Versalovic *et al.* (1994) using the primers and annealing temperature suggested by Belkum & Hermans (2000) [BOXA1R (5'-CTACGGCAAGGCGACGCTGACG-3') and 60 °C].

All PCR reactions were performed in a temperature-gradient thermocycler (Thermo Scientific), and the amplification was carried out with Supreme NZYtaq 2x Green Master Mix (nzytech, Portugal) to which primers (10 µmol/µl), DNA template (75 ng) and ultrapure, sterile water were added to produce a final reaction volume of 25 µl. For BOX-PCR analysis of *L. garvieae* and *S. parauberis* the amplification was carried out with nzytech Supreme NZYtaq DNA polymerase (5U/µl), dNTPs

NZYSets (25 μ M each), $MgCl_2$ (50 nM), primers (10 μ mol/ μ l), DNA template (75 ng) and ultrapure, sterile water were added to produce a final reaction volume of 25 μ l. After amplification samples were kept at 4 °C until analyzed. Electrophoresis was performed as previously described.

All strains of *L. garvieae* were further characterized using Pulsed-Field Gel Electrophoresis (PFGE). An initial study using 10 different restriction enzymes (*Xba*I, *Not*I, *Ssp*I, *Eco*RI, *Kpn*I, *Xba*I, *Ban*I, *Avr*II, *Apa*I, *Sma*I) (Invitrogen, USA), one *L. garvieae* strain (NFM1.07.05) and two different optical densities ($OD_{610\text{ nm}}$: 1.2 and 1.0) was performed to determine the enzyme/OD that originated the best band pattern. Afterwards all strains were characterized using the enzyme *Sma*I and an OD of 1.0. Briefly, *L. garvieae* cells were grown aerobically in TSA-1 at 25 °C for 24 h. After incubation, cells were suspended in 4 ml TE (pH 8.0) and their OD_{610} adjusted to approximately 1.0; 240 μ l of this suspension were transferred to a microcentrifuge tube to which 60 μ l of lysozyme (10 mg/ml) were added. This suspension was incubated in a shaking water bath for 10 minutes at 37 °C. After incubation, agarose plugs were made from a 1:1 mixture of SSP solution [30 μ l SDS 10 %; 267 μ l of 1,2 % agarose; 3 μ l proteinase K (20 mg/ml)] and the cell suspension. The plugs were lysed in 4 ml of lysis buffer [Tris-HCl 1M, pH 8.0; EDTA 0,5M, pH 8.0; 10 % sarcosyl; 30 μ l proteinase K (20 mg/ml)] in a shaking water bath at 55 °C for 2 h. After incubation, plugs were washed twice with pre-heated (55 °C) ultrapure sterile water for 10 min, and four times with pre-heated (55 °C) TE buffer for 15 min. All plugs were kept in fresh TE buffer at 4 °C until digestion. *Sma*I (Invitrogen, USA) was used for restriction endonuclease; half a plug was digested with 15 μ l restriction buffer, 134 μ l of ultrapure water and 1 μ l of restriction enzyme for 12 h at 25°C. The fragments were resolved by PFGE with PFGE grade agarose (Conda-Pronadisa, Spain) by using a CHEF-DR III System (Bio-Rad, USA). The following parameters were used: running time, 20 h; temperature, 14 °C; voltage gradient, 6 v/cm; initial pulse time, 4 s; final pulse time, 40 s; included angle, 120 °. Gels were stained with ethidium bromide (0.5 μ g/ml) for 30 min, destained twice in distilled water for 30 min, and photographed under UV light. An *Xba*I digestion of *Salmonella* Braenderup was used as a ladder for molecular size determination.

12. Computer data analysis

All digitalized images were analysed and processed using Phoretix1D Pro (v12.2, TotalLab, UK), and computed similarities among strains were estimated by means of the Dice coefficient (Dice, 1945). A band position tolerance value of 5 % was allowed to compensate for misalignment of homologous bands due to technical imperfections.

Cluster analysis was performed and dendograms produced following the Unweighted Pair Group Method of Arithmetic averages (UPGMA).

13. Phage isolation and characterization

13.1. Bacteria and media

Bacterial strains used in phage studies are the same as those used in the *A. salmonicida* ssp. *salmonicida* and *L. garvieae* characterization (Tables 2, 3) and the ones on Table 5. Tryptic Soy Broth (Merck) supplemented with 1 % (w/v) NaCl (TSB-1) and with 6 % (Top agar) or 12 % agar (TSA-1) were used for bacterial culture and phage Plaque Forming Units (PFU) assay.

13.2. Phage isolation

Phage isolation was attempted from diseased fish and culture pond water obtained from several fish farms located in Portugal and Spain for several years (2006 to 2011). Phage isolation was also performed from different urban sewage treatment plants and from the Douro river water and from mussels to be headed for human consumption. In all samples, phage were isolated using an enrichment method: for water samples, 36.6 ml of water were filtered through a 0.22 µm pore-size membrane filter (Millipore, USA), mixed with 4 ml of 10x TSB-1 and 400 µl of a day culture of the bacterial strain. If sediments were present, water was centrifuged at 3,000 g for 10 min before filtration. Samples from fish (kidney, spleen or liver) were pooled from three fish and homogenized with sterile saline, centrifuged at 3,000 g for 10 min and subjected to the same procedure as previously described.

After 24 and 48 h of growth at 25 °C with gentle agitation, 1 ml of the culture was

Table 5 - Place of isolation, number of strains of each bacterial species used in phage isolation work.

Bacterial species	Number of Strains	Place of isolation
<i>Streptococcus parauberis</i>	8	Portugal South Korea NCDO 2020
<i>Vibrio anguillarum</i>	8	Portugal Spain Holland
<i>Aeromonas hydrophila</i>	2	Portugal
<i>Yersinia ruckeri</i>	8	Portugal
<i>Escherichia coli</i>	2	Laboratory strains
<i>Vibrio parahaemolyticus</i>	2	Laboratory strains
<i>Photobacterium damsela</i> ssp. <i>piscicida</i>	2	Portugal
<i>Tenacibaculum maritimum</i>	2	Portugal

centrifuged and the supernatant was subjected to the double agar layer technique (Adams, 1959), followed by multiple purification steps using this same technique to identify specific, unique phages for each bacterial pathogen. All plates were incubated at 25 °C.

13.3. Phage stocks

After purification of each phage, stock suspensions were prepared using the double agar layer technique at a phage concentration where confluent lyse was observed. After incubation, 3 ml of SM Buffer [10 mM NaCl; 1 mM MgSO₄; 50 mM Tris-HCl (1 M at pH 7.5); 0.1 g gelatin] were poured and plates were gently agitated for 3 h at 4 °C. After this period, the liquid was removed, centrifuged twice for 20 min at 5,000 G to pellet bacterial debris and filtered through a 0.22 µm syringe filter (Millipore). Phage stock suspensions were stored at 4 °C or at -80 °C in a 50 % glycerol (v/v) solution.

For high volumes of phage stocks, a liquid amplification was prepared in which 500 ml of a bacterial culture in log phase were infected with the phage to be amplified at a multiplicity of infection (MOI) of 0.01. After incubation, 5 ml of chloroform were added and the culture was maintained overnight at 4 °C with gentle agitation to lyse the bacterial cells. Afterwards the culture was centrifuged twice at 5,000 g for 20 min to pellet bacterial debris, and the supernatant was centrifuged for 2 h at 10,000 g to pellet phage. After removing the supernatant, 5 ml of SM buffer were added to the pellet and allowed to rest overnight in the dark to allow phages to dissociate from one another. Phage stock solutions were filtered through a 0.22 µm syringe filter, stored at 4 °C or at -80 °C in a 50 % glycerol (v/v) solution.

13.4. Prophage induction

All isolates of *A. salmonicida* ssp. *salmonicida* and *L. garvieae* used in the host range study (Tables 2 and 3) were tested for lysogenic phages using the method described by Fortier & Moineau (2007). In summary, an overnight culture of the bacteria was sub-cultured (3 % v/v) in fresh TSB-1 and incubated at 25 °C with agitation until cultures reached an OD (600 nm) of 0.1. Then Mitomycin C (Sigma- Aldrich, St Louis, MO, USA) was added to a final concentration of 1 µg/ml, incubated at 25 °C and the OD (600 nm) was monitored regularly. A significant decrease in the cell density after 3 to 5 h suggested that prophages were released.

13.5. Determination of phage host range

Lawns of the trial bacteria (Tables 2, 3 and 5) were produced by pipetting 600 µl of a day culture onto 6 ml of top agar and poured over a square plate containing a basal layer TSA-1, allowed to cover the entire plate and solidify. This method provided an even covering of bacteria producing consistent lawns. 5 µl of each phage stock were pipetted onto lawns of each trial bacterial strain to form distinct spots. Spotted lawns were incubated at 25 °C for 24 h and then inspected for zones of clearing.

13.6. Phage purity and genome size determination using PFGE.

For preparation of phage genome, 400 µl of a 10⁹ PFU/ml suspension of each phage were mixed with an equal volume of 1.4 % (w/v) molten PFGE agarose in TE buffer and dispensed into plug molds, which were allowed to set at room temperature. Molds were transferred to a Falcon tube containing 2 ml of lysis buffer (1 % N-lauryl sarcosine; 0.2 % SDS; 0.25 mg/ml Proteinase K) and incubated overnight at 55 °C in a shaking (100-200 rpm) water bath. Plugs were then washed with 2 ml of wash buffer (20 mM Tris-HCl; 50 mM EDTA t pH 8.0) for 1 hr at room temperature and 3 times (30 min per wash) in TE buffer (10 mM Tris-HCl; 1 mM EDTA at pH 8.0). After the last wash, plugs were stored at 4 °C in TE buffer.

Plugs were electrophoresed in a 1 % PFGE grade agarose gel and run on 0.5x TBE buffer (45 mM Tris-Borate; 1 mM EDTA at pH 8.0) with the following conditions: initial switch time - 2 s; final switch time - 10 s; voltage - 6 v/cm; time - 18 h.

The PFGE size standard used was MidRange PFGE Marker I (New England Biolabs, USA). After electrophoresis, gels were stained in ethidium bromide (Sigma, USA) (2 µg/ml) for 30 min, destained twice in distilled water for 30 min, and photographed in a UV transilluminator.

13.7. Analysis of phage nucleic acids

Plugs of phage were tested for sensitivity to DNase and RNase (Bio-Rad). Each plug was digested in 1 ml of a 20 µg/ml DNase or RNase solution for 2 h at 37 °C. Phage sensitivity to various restriction enzymes (*Sma*I, *Bam*HI, *Xba*I, *Xho*I, *Ssp*I, *Avr*II, *Eco*RI and *Not*I) was also tested using the conditions recommended by the supplier (New England Biolabs, USA). The results were determined by 1 % agarose gel electrophoresis in SB buffer.

Results

Distribution of bacterial fish pathogens in a production facility

With very few exceptions, water entering the facility (Table 6; P1) consistently had bacterial loads in the order of 10^3 - 10^4 CFU/ml. Only on June 6 was this load lower (6.5×10^2 CFU/ml) and values in the order of 10^5 CFU/ml were obtained twice (July 21 and September 15).

After entering the facility, water passed through a sand filter (P2) and an UV filter (P3). For several months, bacterial loads detected in P2 were often higher than in P1 (Table 6) indicating that the sand filter appeared to be functioning as a focus of infection. Additionally, the rapid clogging of this filter led to a decrease in the amount of water reaching the tanks. Since the loads in P3 were only less than 1 log lower than in P2 (Table 6) and sometimes higher (in particular, on June 23, when the value obtained was much higher than in P2), it became clear that the UV system was not effective in reducing the bacterial load in the water.

Also, filters intended to improve the bacteriological quality of the water for the hatchery and nursery gave inconsistent results, as shown in Table 6 (P4-P6). Another problem seemed to arise from the skimmer located before reservoir 2 (Fig. 1), as numerous tiny gas bubbles were frequently observed in fingerlings (Fig. 2) and were blamed for some mortality. For this reason, this equipment was taken off the circuit at the end of July 2009; gas bubbles stopped being observed and part of the associated fingerling mortality disappeared.

The high bacterial load observed in the water leaving the nursery tanks (Table 2, P7) was the most unexpected value obtained throughout the facility.

The ozone skimmer (P9) used for treating recirculated water also seemed to be ineffective, as bacterial counts before and after the skimmer were the same (around 10^5 - 10^6 CFU/ml) (Table 6, P8 and P9, respectively).

The physiological and biochemical study of the colonies isolated from MA allowed us to identify the bacteria as belonging to one of the following groups: *Enterobacteriaceae*, *Vibrio* sp., *Aeromonas* sp., *Photobacterium* sp. and *T. maritimum*.

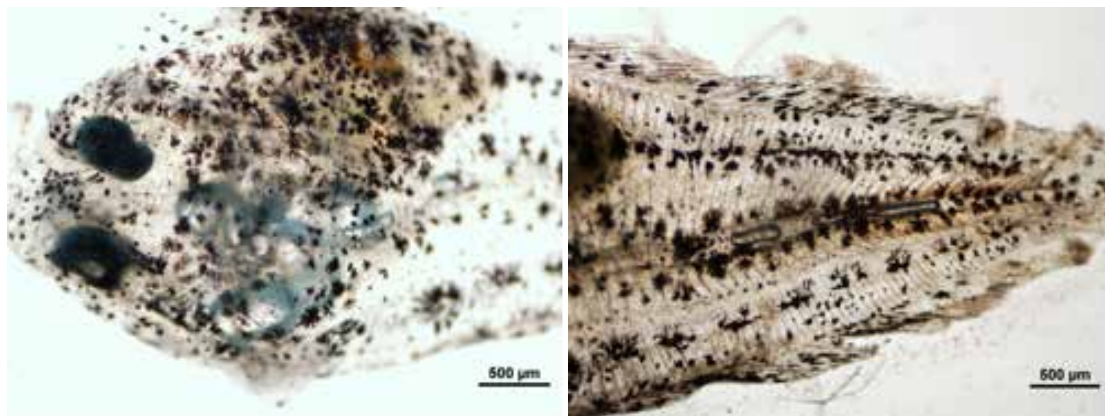


Figure 2 - Gas bubbles observed in the body of larval sole.

Table 6 - Number of bacteria (CFU/ml) detected in the water of different places throughout the facility (Figure 1) during the initial sampling period. Numbers represent average values of duplicate counting of CFU/ml in plates with Marine Agar medium. D - P4 was discontinued after the end of July 2009.

--- Data not available.

Sampling Date	P1	P2	P3	P4	P5	P6	P7	P8	P9
February 2009	2.47X10 ³	6.3X10 ³	9.7X10 ²	6.0X10 ³	6.4X10 ³	4.9X10 ³	7.7X10 ⁴	5.45X10 ⁵	2.8X10 ⁵
April 2009	1.93X10 ⁴	2.13X10 ⁴	8.5X10 ³	9.2X10 ³	7.3X10 ³	2.8X10 ³	3.1X10 ⁴	1.13X10 ⁶	3.3X10 ⁶
May 2009	1.72X10 ³	2.34X10 ³	1.0X10 ²	1.46X10 ³	3.2X10 ³	1.4X10 ⁴	9.7X10 ⁵	4.15X10 ⁶	1.74X10 ⁶
June 6, 2009	6.5X10 ²	8.4X10 ²	0	7.1X10 ²	9.0X10 ¹	6.1X10 ²	4.4X10 ³	---	1.3X10 ⁵
June 23, 2009	8.8X10 ⁴	6.8X10 ⁴	2.79X10 ⁶	3.5X10 ⁴	5.0X10 ⁴	1.7X10 ⁴	7.5X10 ⁴	1.44X10 ⁶	2.49X10 ⁶
July 7, 2009	2.16X10 ⁴	2.64X10 ³	3.09X10 ³	1.95X10 ³	1.63X10 ³	2.25X10 ³	1.8X10 ⁴	1.07X10 ⁶	1.01X10 ⁶
July 21, 2009	2.37X10 ⁵	6.9X10 ⁴	1.32X10 ⁵	8.2X10 ⁴	7.2X10 ⁴	6.6X10 ⁴	1.2X10 ⁴	---	---
July 27, 2009	3.28X10 ³	2.3X10 ³	3.41X10 ³	2.41X10 ³	3.78X10 ³	6.0X10 ³	---	4.15X10 ⁶	1.74X10 ⁶
August 3, 2009	1.75X10 ³	1.6X10 ³	2.0X10 ³	D	8.0X10 ²	1.0X10 ³	8.4X10 ⁵	8.8X10 ⁵	1.28X10 ⁶
August 17, 2009	3.8X10 ³	1.39X10 ⁴	8.7X10 ³	D	2.57X10 ³	1.73X10 ³	4.5X10 ⁴	9.3X10 ⁵	1.11X10 ⁶
August 25, 2009	4.6X10 ³	4.3X10 ³	3.8X10 ³	D	2.7X10 ³	2.07X10 ³	1.08X10 ⁵	1.87X10 ⁶	---
September 1, 2009	3.75X10 ³	3.45X10 ⁴	3.25X10 ³	D	1.9X10 ³	2.6X10 ³	1.6X10 ⁴	1.68X10 ⁶	1.38X10 ⁶
September 8, 2009	2.72X10 ³	2.95X10 ³	2.61X10 ³	D	2.07X10 ³	2.0X10 ⁴	1.95X10 ⁴	2.12X10 ⁶	1.0X10 ⁶
September 15, 2009	2.15X10 ⁵	1.3X10 ⁵	2.04X10 ⁵	D	1.58X10 ⁵	2.16X10 ⁴	3.9X10 ⁴	4.0X10 ⁵	1.4X10 ⁵
September 23, 2009	5.10X10 ³	6.60X10 ³	3.69X10 ³	D	6.90X10 ³	1.58X10 ³	1.40X10 ⁴	1.18X10 ⁶	2.76X10 ⁵

However, with the exception of *T. maritimum*, none of the other bacteria were identified as a fish pathogen by PCR.

Many colonies obtained from water showed phenotypical and biochemical/physiological characteristics similar to those described by Austin & Austin (2012) for the fish pathogenic bacteria searched (particularly the Gram-negative, as very few presumptive *Streptococcaceae* were detected). However, only *T. maritimum* was confirmed by PCR to be present in fish tissues and in water samples (Table 7).

No *Streptococcaceae* was observed in sole, and among the Gram-negative a number of presumptive vibrios (possibly *V. anguillarum*) and *T. maritimum* were isolated. From these, only 15 isolates were confirmed by PCR as *T. maritimum* (Table 7). From the DNA extracted from fish mucus only *T. maritimum* DNA was detected in it (Table 7).

Table 7 - Detection of *Tenacibaculum maritimum* during the present study: numbers of isolates obtained from fish and from water, and results from mucus analysis by PCR.

Sampling	Nº of <i>T. maritimum</i> isolates from		<i>T. maritimum</i> detection in fish mucus by PCR
	Fish	Water	
October 2008	2	0	+
February 2009	4	0	+
April 2009	0	0	-
May 2009	0	0	-
June 2009 *	4 ^{a)}	4 ^{b)}	+ ^{g)}
July 2009 *	0	2 ^{c)}	-
August 2009 *	0	0	+ ^{h)}
September 2009 *	5 ^{d)}	4 ^{e)}	+ ⁱ⁾
October 2009	0	0	-
November 2009	0	6 ^{f)}	+
December 2009	0	0	+
January 2010	0	0	+
March 2010	0	0	+
April 2010	0	0	-
May 2010	0	0	-

* Includes all samplings effectuated in this month (as indicated in Table 6);

^{a)} All 4 isolates obtained in June 16;

^{b)} 1 isolate obtained in June 6 and 3 in June 16 (from 4 different tanks);

^{c)} Both isolates obtained in July 7 (from different tanks);

^{d)} All 5 isolates obtained in September 15

^{e)} 1 isolate obtained in September 1 and 3 in September 15 (from 4 different tanks);

^{f)} Isolates obtained from 6 different sampling places;

^{g)} *T. maritimum*'s DNA detected in June 6 and June 16;

^{h)} *T. maritimum*'s DNA detected only in August 25;

ⁱ⁾ *T. maritimum*'s DNA detected in September 8 and September 15.

As stated above, *T. maritimum* was the only pathogen already known to be present in the facility and, in the present study, a number of isolates were obtained both from water and from sole with signs of disease, and its DNA was frequently detected in fish mucus. These results led us to suspect that the pathogen was widespread in the facility. To test this suspicion, a thorough molecular surveillance was undertaken between October 2009 and May 2010 in several tanks located throughout the farm. DNA was isolated from water, sediment and/or biofilm and used for the molecular detection of *T. maritimum* and the other bacterial fish pathogens following the PCR protocols used with mucus samples.

As with the results obtained in the isolation trials and PCR study of fish mucus, positive results were only obtained for *T. maritimum* (Table 8). This pathogen's DNA was detected in water and sediment/biofilm samples of all tanks tested, confirming our suspicion that it was clearly widespread throughout the facility.

Table 8 - Detection of *Tenacibaculum maritimum* by PCR in samples of water and sediment/biofilm. + *T. maritimum* detected; - *T. maritimum* not detected. PE, T4, T5, T11, T16 and TD – different tanks (numbers in the facility); NT – not tested.

Sampling	Water						Sediment / Biofilm					
	PE	T4	T5	T11	T16	TD	PE	T4	T5	T11	T16	TD
October 2009	-	-	+	+	-	+	-	-	+	-	+	+
November 2009	+	-	+	-	+	-	+	+	+	-	+	+
December 2009	-	+	+	-	-	+	-	+	-	+	-	+
January 2010	+	-	NT	+	+	NT	+	+	NT	+	+	NT
March 2010	+	-	+	+	+	+	+	-	+	+	+	+
April 2010	-	+	-	NT	+	+	+	+	+	NT	+	+
May 2010	+	NT	+	-	+	+	+	NT	+	+	+	+

Characterization of Portuguese strains of *Aeromonas salmonicida* ssp. *salmonicida*

Biochemical characterization showed that all isolates of *Aeromonas salmonicida* ssp. *salmonicida* from Portugal, Spain and the US formed a very homogeneous group (Table 9). The biggest variation was observed in arginine dihydrolase (ADC), where 58 % of the Portuguese isolates showed a negative reaction (Table 9). All strains were able to grow at 4 °C, 22 °C and 30 °C, with 0 to 3 % salt, but none was able to grow at 37 °C.

When using the API 20E system with the Portuguese strains, four different

Table 9 - Biochemical characteristics of the *Aeromonas salmonicida* ssp. *salmonicida* used in this study. Number between brackets represents the percentage of isolates that gave the result. (+) positive result; (-) negative result.

Character	Place of origin					
	Portuguese strains			North American strains	Spanish strains	Type strain
	Farm IN	Farm CA	Farm SRab			
Gram stain	-	-	-	-	-	-
Motility	-	-	-	-	-	-
Oxidase	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Pigment production	+	+	+	+	+	+
Citrate	-	-	-	-	-	-
Indole	-	-	-	-	-	-
H ₂ S production	-	-	-	-	-	-
Voges-Proskauer	-	-	-	-	-	-
Methyl-Red	+	+	+	+	+	+
β -Galactosidase	+	+	+	+	+	+
Urease	-	-	-	-	-	-
Hydrolysis of:						
Arginine	+ (50%)	+ (48%)	+	+	+	+
Gelatin	+	+	+	+	+	+
Decarboxylation of:						
Lysine	+	+	+	+	+	+
Ornithine	-	-	-	-	-	-
Growth in:						
0 % NaCl	+	+	+	+	+	+
3 % NaCl	+	+	+	+	+	+
Growth at:						
4 °C	+	+	+	+	+	+
22 °C	+	+	+	+	+	+
30 °C	+	+	+	+	+	+
37 °C	-	-	-	-	-	-
Acid from:						
Amygdalin	-	-	-	-	-	-
Arabinose	-	-	-	-	-	-
Inositol	-	-	-	-	-	-
Maltose	+	+	+	+	+	+
Mannose	+	+	+	+	+	+
Melibiose	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-
Sacarose	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-

numerical profiles were obtained (0006104, 1006104, 2006104 and 3006104) all of which identified as *A. salmonicida* ssp. *salmonicida* in the API 20E Web V4.1. The American strains originated two different numerical profiles (3007104 and 2007104), with the first one identified as *A. hydrophila/caviae/sobria* and the other one as *A. salmonicida* ssp. *salmonicida*.

With the API 20NE system seven different numerical profiles were obtained (5474304, 5474344, 5474744, 5474754, 5574744, 5574754 and 5574764), all identified in the API 20NE Web V7.0. Three of these profiles (5474744, 5574744 and 5574764) identified the isolates as *A. salmonicida* ssp. *salmonicida*; one (5474344) as *A. salmonicida* ssp. *salmonicida* or *Vibrio alginolyticus* with 80.2 % and 19.0 % respectively; two other (5474754 and 5574754) as *A. hydrophila/caviae* with the possibility of *A. salmonicida* ssp. *salmonicida*, with 7.7 % and 1.3 % respectively; the last profile (5474304) identified the isolates as *Vibrio vulnificus*. The American strains originated two different numerical profiles (5474754 and 5574744); the first one was identified as *A. hydrophila/caviae* with the possibility of *A. salmonicida* ssp. *salmonicida* and the other one as *A. salmonicida* ssp. *salmonicida*.

All strains were serologically identified with the BIONOR MONO-AS kit.

The susceptibility pattern of the bacterial isolates to the nine antimicrobial drugs tested is shown in Table 10. Most of the Portuguese strains were susceptible to oxolinic acid, chloramphenicol and nalidixic acid, and resistant to streptomycin, sulphonamids and tetracycline, showing a different degree of sensibility to nitrofurantoin, ampicillin, oxytetracycline and tetracycline

When comparing the percentage of strains that showed sensitivity/resistance to the different antibiotics, there are differences in the percentages against nitrofurantoin, oxytetracycline, ampicillin and tetracycline, however these differences are bigger

Table 10 - Percentage of resistance in the Portuguese strains of *Aeromonas salmonicida* ssp. *salmonicida* isolated from different farms.

Resistance/Sensitivity to:	Percentage of resistant strains		
	Farm IN	Farm CA	Farm SRab
Ampicillin	63	41	80
Chloramphenicol	0	2	0
Nalidixic Acid	1	7	0
Nitrofurantoin	89	58	80
Oxolinic acid	3	7	0
Oxytetracycline	10	87	0
Streptomycin	99	98	100
Sulphonamide	97	98	100
Tetracycline	7	57	0
Trimethoprim	91	98	100

between farms IN and SRab or CA and SRab, than between IN and CA (Table 10).

One strain from farm CA was not identified as *A. salmonicida* ssp. *salmonicida* when using the AP primers, however, when using the MIY primers all the strains gave the expected PCR amplification product.

The plasmid profiles obtained with the Kado & Liu (1981) method were different from the Birnboim & Doly (1979) method. With the Kado & Liu (1981) method four different plasmid profiles were obtained (Figure 3). In all profiles, three small plasmids of 3.4, 3.5 and 3.6 Kb were present. The most common profile, profile I, had the three small plasmids and another of 7.5 Kb. In profile II, only the three small plasmids were present. This profile was obtained with two Portuguese strains (CA1.1.99 and CA4.1.99) and one Spanish (Isla 2002). In profile III, besides the three small plasmids, another of 4.2Kb was present. This third profile was obtained with all Portuguese strains from farm SRab, and with the type (ATCC 14174) and one Spanish strain (ACR 173.1). In the fourth profile (profile IV) we could observe the same four plasmids of profile I and another of 13 Kb. This profile was obtained with two Portuguese strains (CA6.8.99 and CA6.14.99).

With the method of Birnboim & Doly (1979) only a profile was obtained. In this profile nine different plasmids were observed (Figure 4); a first group of three small plasmids of 3.4, 3.5 and 3.6 Kb, a second group of three small plasmids of 5.2, 5.4

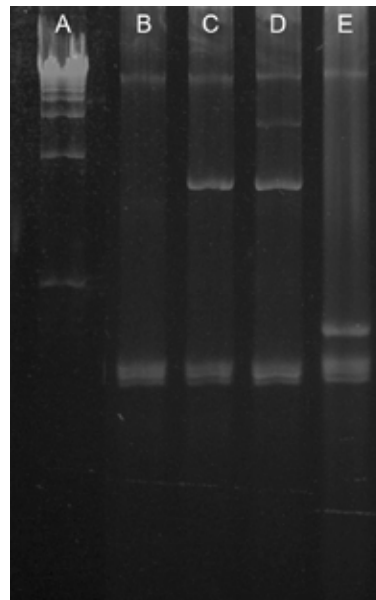


Figure 3 - The four plasmid profiles obtained with the Portuguese strains of *Aeromonas salmonicida* ssp. *salmonicida*, using the Kado & Liu (1981) extraction method. Lane A - 0,05 - 1M basepair (bp) DNA mass marker (Bio-Rad, USA); Lane B - Plasmid profile II; Lane C - Plasmid profile I; Lane D - Plasmid profile IV; Lane E - Plasmid profile III.

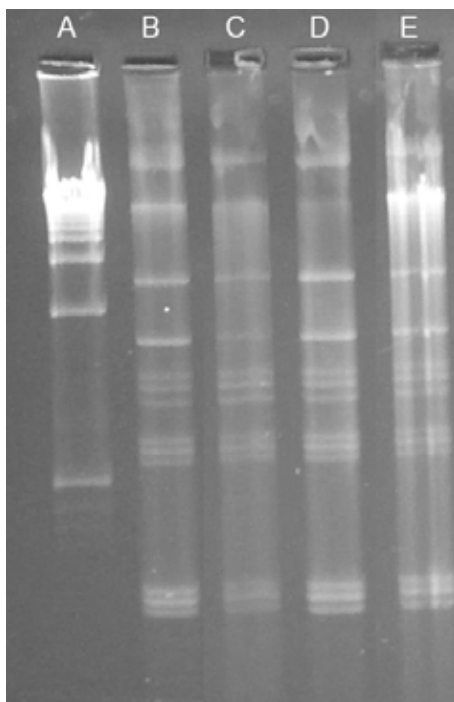


Figure 4 - Plasmid profiles obtained with the Portuguese strains of *Aeromonas salmonicida* ssp. *salmonicida*, using the Birnboim & Doly (1979) extraction method. Lane A - 0,05 - 1M basepair (bp) DNA mass marker (Bio-Rad, USA); Lane B, C, D, E - plasmid profiles.

and 5.6 Kb, and a third group of plasmids of 5.7, 6.0 and 6.3 KB. Finally, three other plasmids of 7.5 and 11.2 Kb could also be observed.

Using the primer P4 of the Ready-To-Go RAPD Analysis Beads (GE Healthcare) we were able to type the DNA of all the *A. salmonicida* ssp. *salmonicida* strains used in this study revealing a typeability of 100 %. With this technique 11 different DNA

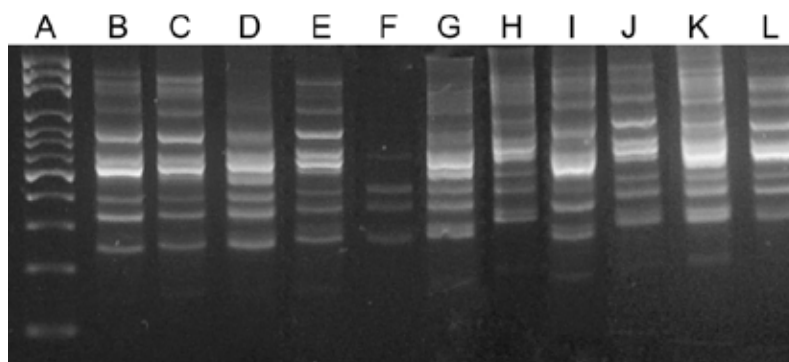


Figure 5 - The 11 different DNA fragment profiles, obtained with primer P4 of the Ready-To-Go RAPD Analysis Beads (GE Healthcare, UK) in *Aeromonas salmonicida* ssp. *salmonicida*. Lane A - 100 bp Molecular weight ladder (Solis BioDyne, Estonia); Lane B - RAPD type K; Lane C - RAPD type B; Lane D - RAPD type A; Lane E - RAPD type J; Lane F - RAPD type F; Lane G - RAPD type H; Lane H - RAPD type D; Lane I - RAPD type E; Lane J - RAPD type I; Lane K - RAPD type G; Lane L - RAPD type C.

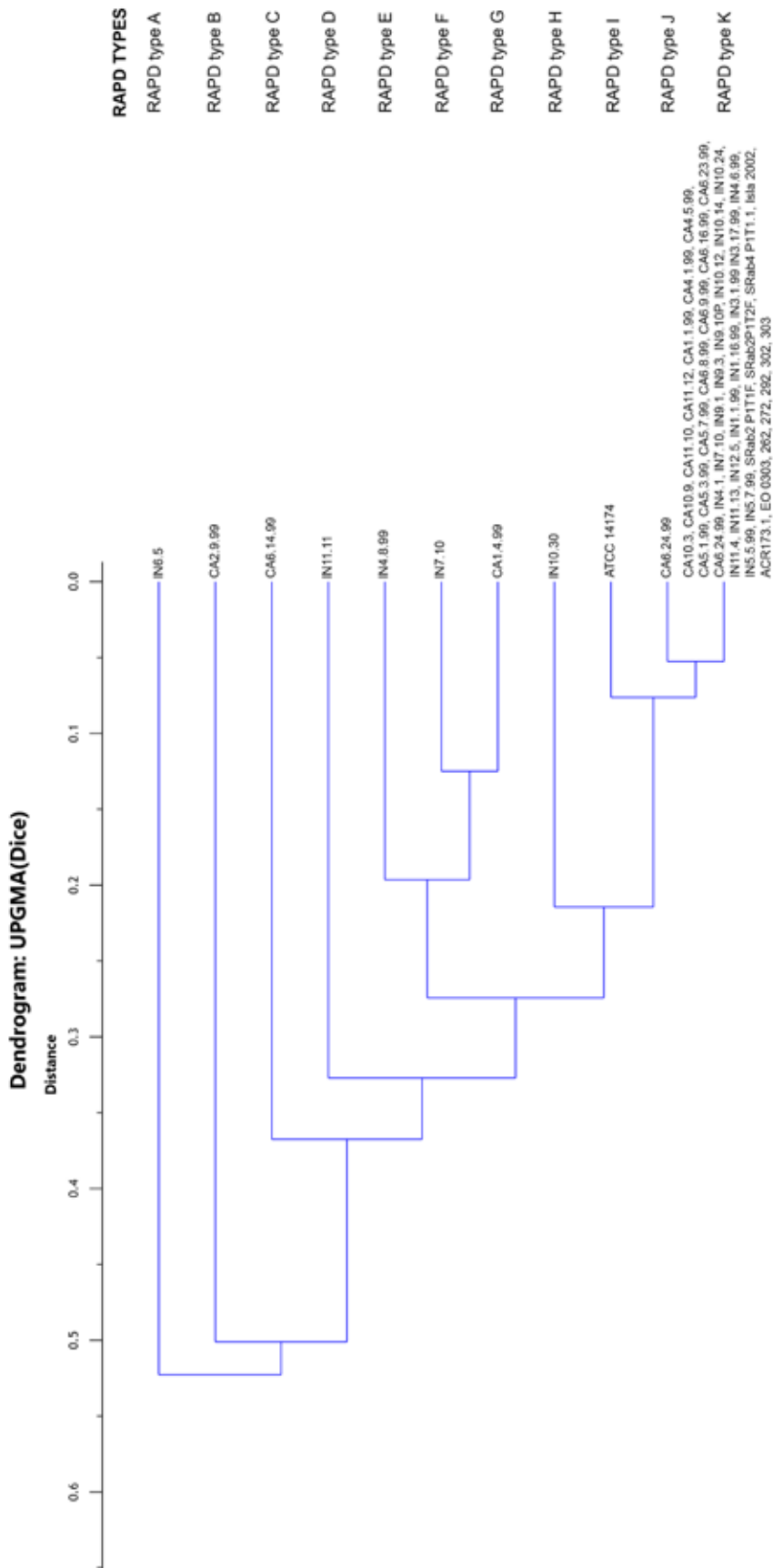


Figure 6 - Dendrogram established by Phoretix1D Pro software package (TotalLab, UK) using the Dice similarity coefficient and UPGMA on the basis of the RAPD profiles of *Aeromonas salmonicida* ssp. *salmonicida* strains obtained with primer P4 of the Ready-To-Go RAPD Analysis Beads (GE Healthcare, UK).

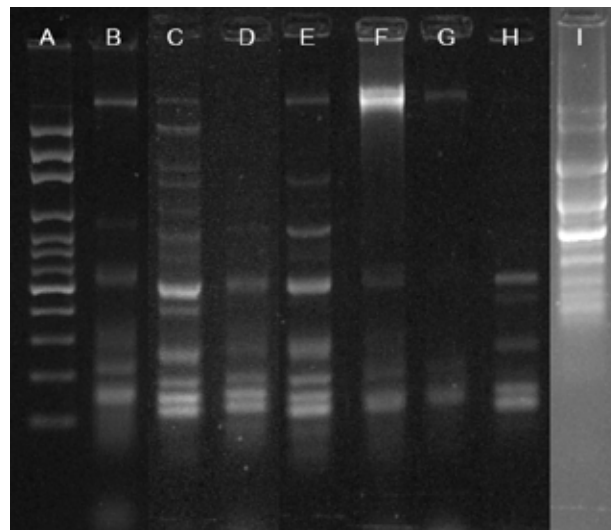


Figure 7 - The eight different DNA fragment profiles, obtained in *Aeromonas salmonicida* ssp. *salmonicida* with the REP-PCR technique using the primers REP1R-I and REP2-I. Lane A - 100 bp Molecular weight ladder (Solis BioDyne, Estonia); Lane B - REP type H; Lane C - REP type D; Lane D - REP type G; Lane E - REP type C; Lane F - REP type F; Lane G - REP type B; Lane H - REP type E; Lane I - REP type A.

fragment profiles were established (RAPD types A to K) (Figure 5). After computer analysis (Figure 6) we could observe that 80 % of all the Portuguese strains clustered together on a single RAPD type. In this RAPD type were also included all the American and Spanish strains. The remaining 9 Portuguese strains produced nine different RAPD types.

With the application of the REP-PCR technique, with primers REP1R-I and REP2-I, we were able to type all *A. salmonicida* ssp. *salmonicida* strains used in this study revealing a typeability of 100 %. With this technique 8 different DNA fragment profiles, designated REP types A to H were established. The variability observed with this technique was due to the presence of bands with more than 600 bp (Figure 7). After computer analysis (Figure 8) we could observe that 82 % of the Portuguese isolates were clustered together on a single REP type. This REP type also included the American and two of the three Spanish strains (Figure 8). Two other REP types with two strains each could be established; one with two strains from the farm CA from the same month and the second one with one strain from farm IN and one Spanish strain.

Using the ERIC-PCR technique, we were also able to type the DNA of all the *A. salmonicida* ssp. *salmonicida* strains used in this study. With this technique 4 different DNA fragment profiles were established (ERIC types A to D). Using this technique all strains produced a band of 50 and 100 bp (Figure 9). After computer analysis (Figure 10) we could observe the presence of two ERIC types: one containing all the strains from farm SRab and two strains each from farm IN and CA (this ERIC type

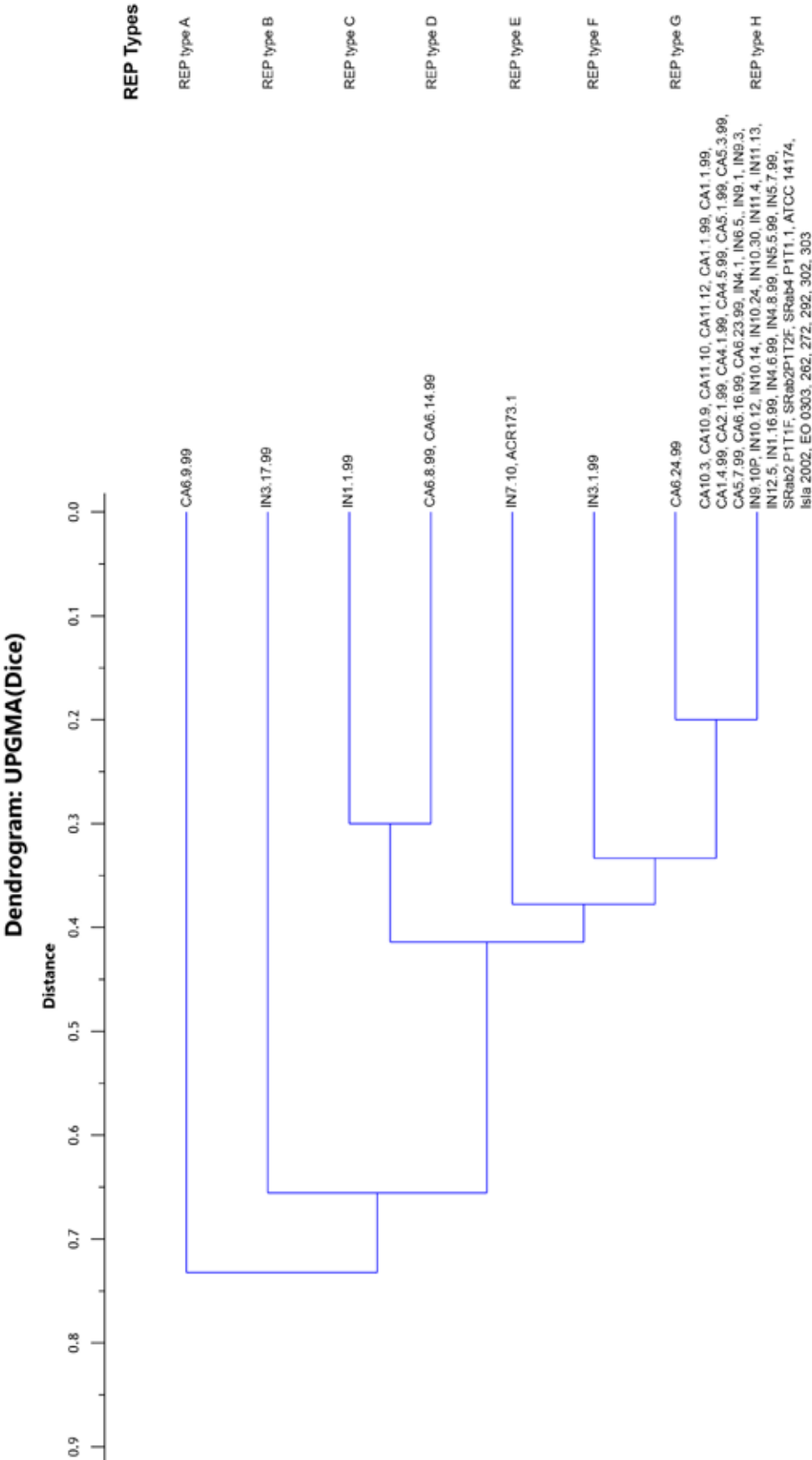


Figure 8 - Dendrogram established by Phoretix1D Pro software package (TotalLab, UK) using the Dice similarity coefficient and UPGMA on the basis of the REP profiles obtained with *Aeromonas salmonicida* ssp. *salmonicida* using the primers REP1R-I and REP2-I.

represented 15.5 % of the Portuguese strains); the other ERIC type contained all but one Portuguese and Spanish strains and all the American strains. However, due to their similarity, all strains with the exception of one Spanish strain (ACR173.1) could be joined together in a cluster group at a distance of 0.2.

With BOX-PCR, all *A. salmonicida* ssp. *salmonicida* were typeable. The profiles of the 54 isolates showed multiple DNA bands corresponding to sizes ranging from approximately 300 to 3000 bp, with the exception of strain IN7.10 that showed only one band (Figure 11). With this technique 7 different DNA fragment profiles, designated BOX types A to G (Figure 12) were established: one of the BOX types contained all but two Portuguese strains and one Spanish strain; another contained three of the American strains and the type strain; and the third one contained the remaining Spanish strains. The remaining two American strains and the Portuguese strains formed their own BOX types.

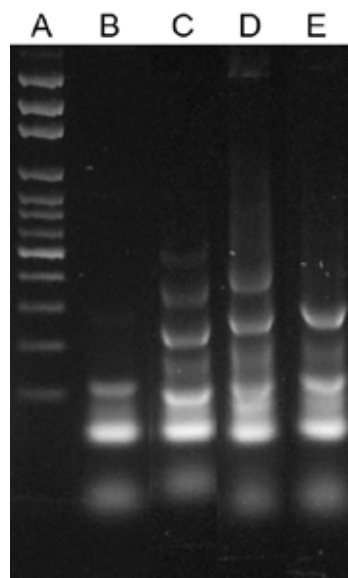


Figure 9 - The four different DNA fragment profiles in *Aeromonas salmonicida* ssp. *salmonicida* obtained with the ERIC-PCR technique. Lane A - 100 bp Molecular weight ladder (Solis BioDyne, Estonia); Lane B - ERIC type A; Lane C - ERIC type B; Lane D - ERIC type C; Lane E - ERIC type D.

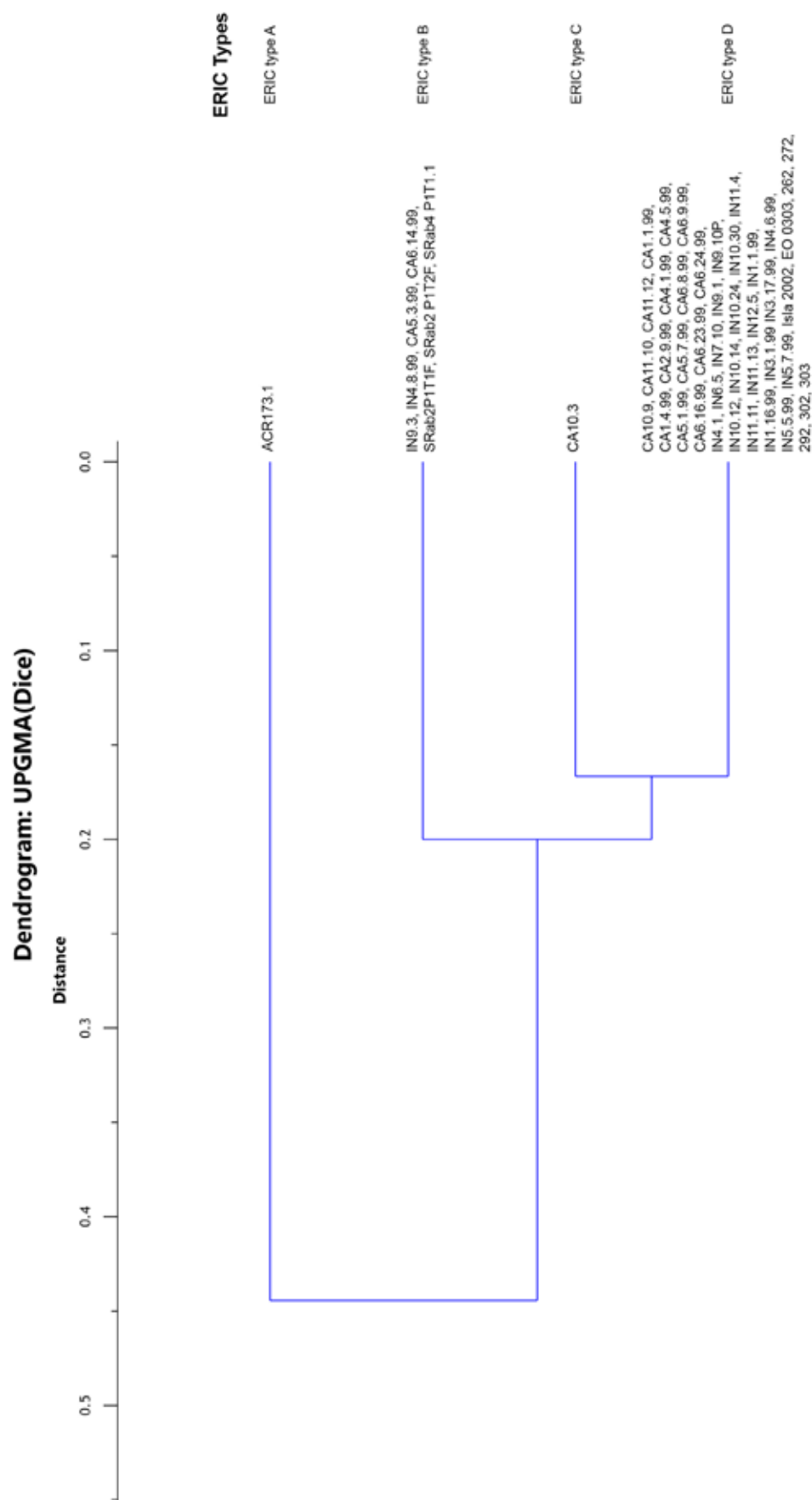


Figure 10 - Dendrogram established by Phoretix1D Pro software package (TotalLab, UK) using the Dice similarity coefficient and UPGMA on the basis of the ERIC-PCR profiles obtained with *Aeromonas salmonicida* ssp. *salmonicida*.

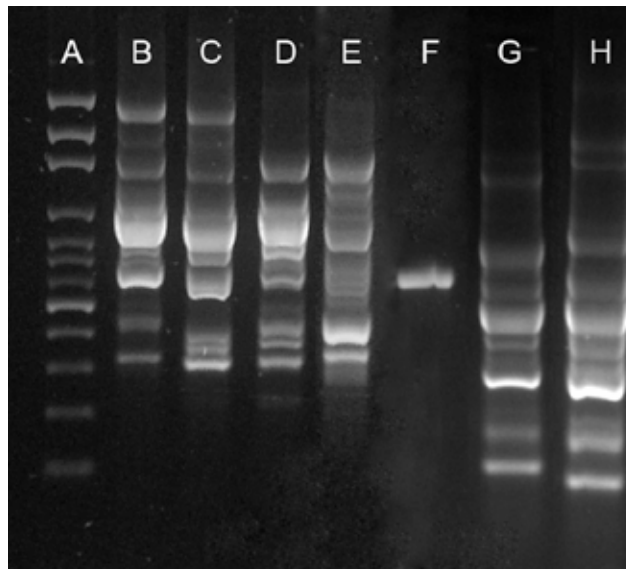


Figure 11 - The seven different DNA fragment profiles obtained in *Aeromonas salmonicida* ssp. *salmonicida* with the BOX technique using primer BOXA1R. Lane A - 100 bp Molecular weight ladder (Solis BioDyne, Estonia); Lane B - BOX type D; Lane C - BOX type C; Lane D - BOX type G; Lane E - BOX type A; Lane F - BOX type E; Lane G - Box type F; Lane H - BOX type B.

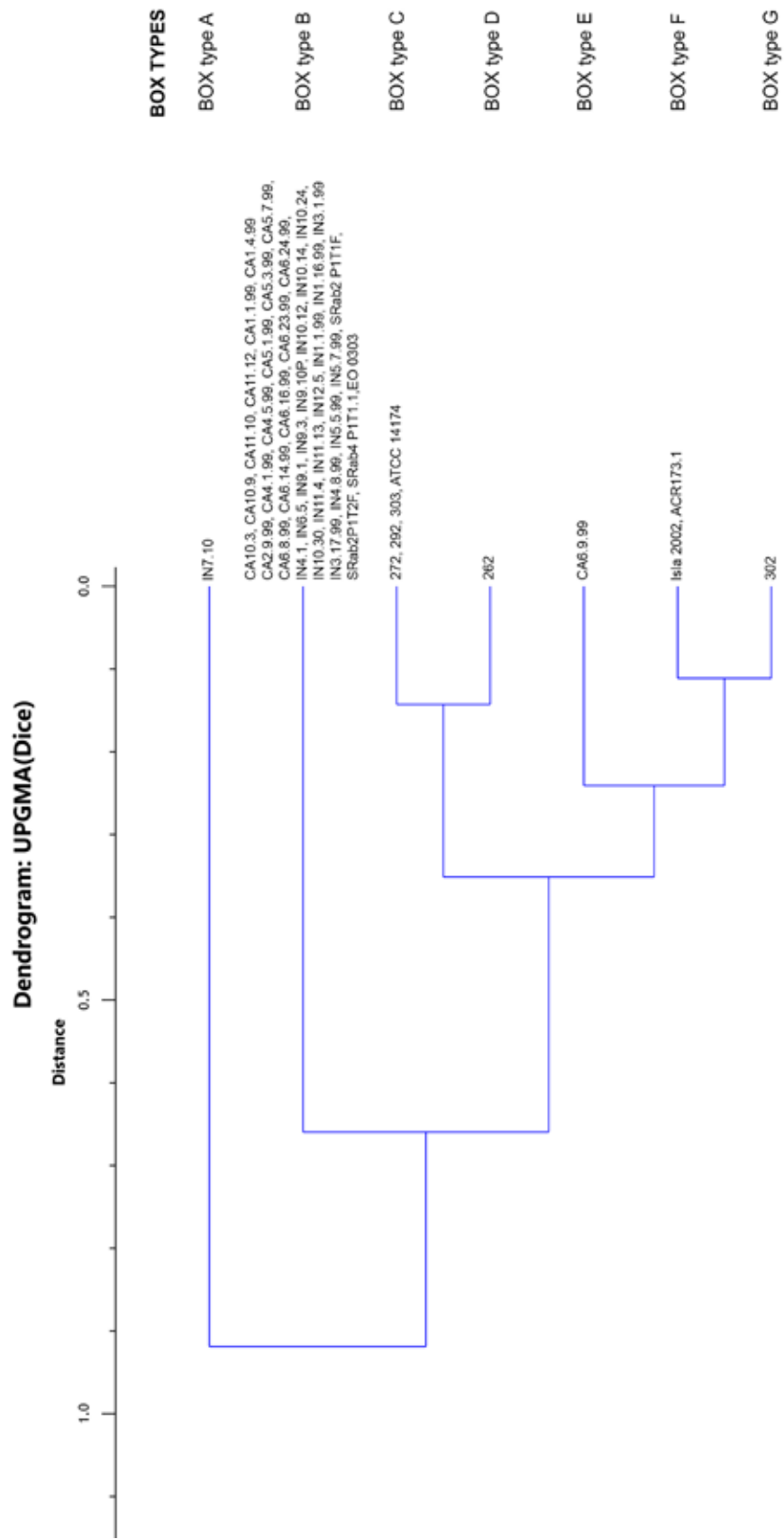


Figure 12 - Dendrogram established by Phoretix1D Pro software package (TotalLab, UK) using the Dice similarity coefficient and UPGMA on the basis of the BOX profiles obtained with *Aeromonas salmonicida* ssp. *salmonicida* using the primer BOXA1R..

Characterization of Portuguese strains of *Lactococcus garvieae*

During visits to five trout farms in the north of Portugal we could observe that some fish showed melanosis, pronounced uni- or bilateral exophthalmia with abscesses and periocular haemorrhages, with loss of eyes in extreme cases (Figure 13). Frequently, fish showed erosion and/or haemorrhages of the anal, dorsal and caudal fins and haemorrhages in the mouth and operculum. Internally, clinical signs consisted of splenomegaly, accumulation of ascitic fluid in the peritoneal cavity and petechial haemorrhages in the liver, intestine and muscle. Fish of all sizes were affected, from 0.9 g to 581.5 g, although mortalities were heavier in bigger fish.

From sterile swabs, growth was observed on TSA-1 and BA medium from all examined organs, frequently in multiple organs and in pure culture. Pinpoint colonies 1-2 mm in diameter, raised, grayish-white with entire margin and α -haemolysis producers were detected in BA after 24-48 h incubation at 25 °C. In TSA-1, pinpoint colonies with entire margin, 1-3 mm in diameter, raised and white were observed. A total of 34 isolates obtained preferably from the kidney were used in the present study.

Regardless of their origin, all isolates obtained from the sampled fish were Gram-positive, non-motile cocci that occurred singly or in short chains that did not produce oxidase or catalase. With the standard morphological, physiological and biochemical plate and tube tests, all isolates were facultative anaerobic, citrate, Voges-Proskauer, urea, indole, hydrogen sulphide (H_2S), gelatinase and ortho-nitrophenyl- β -galactoside (ONPG) negative. In addition, isolates possessed the enzyme arginine dihydrolase but not lysine or ornithine decarboxylase. All strains were methyl-red (MR) and nitrate positive and were able to produce acid from salicin, galactose and mannose but not from mannitol, maltose and lactose (Table 11).



Figure 13 - Rainbow trout with bilateral exophthalmia and rupture of eye, typical signs of *Lactococcus garvieae*.

Table 11 - Biochemical characteristics of the *Lactococcus garvieae* used in this study; (+) positive result; (-) negative result.

Character	Strains		
	Portuguese strains (n=34)	Spanish Strain (n=1)	Type strain NCDO 2155
Gram stain	+	+	+
Motility	-	-	-
Oxidase	-	-	-
Catalase	-	-	-
Pigment production	-	-	-
Haemolytic type	α	α	α
O/F	F	F	F
Indole	-	-	-
H ₂ S production	-	-	-
Voges-Proskauer	-	-	-
Methyl-Red	-	-	-
Nitrates	+	+	+
ONPG	-	-	-
Gelatinase	-	-	-
Urea	-	-	-
Hydrolysis of:			
Arginine	+	+	+
Decarboxylation of:			
Lysine	-	-	-
Ornithine	-	-	-
Growth at:			
4 °C	+	+	+
35 °C	+	+	+
40 °C	+	+	+
Growth in:			
1 % NaCl	+	+	+
2 % NaCl	+	+	+
4 % NaCl	+	+	+
6.5 % NaCl	+	+	+
Acid from:			
Mannitol	-/-	-/-	-/-
Maltose	-/-	-/-	-/-
Salicin	+/+	+/+	+/+
Galactose	+/+	+/+	-/-
Lactose	-/-	-/-	-/-
Mannose	+/+	-/-	+/+

When using the miniaturized system developed by Carson *et al.* (2001) we obtained the same biochemical results, with exception of nitrates that in this system were negative.

The use of the API 20Strep system with the Portuguese isolates, after a 4 h

incubation period, showed that all strains were negative to α -galactosidase (α GAL), β -glucuronidase (β GUR), β -Galactosidase (β GAL), alkaline phosphatase (PAL), arabinose (ARA), sorbitol (SOR), lactose (LAC), inulin (INU), raffinose (RAF), starch (AMD) and glycogen (GLYG). Voges-Proskauer (VP), esculin (ESC), L-leucine-2-naphthylamide (LAP), trehalose (TRE) were positive, and pyrrodidonyl arylamidase (PYRA), arginine dihydrolase (ADH), ribose (RIB) and mannitol (MAN) showed variable results. Hippuric acid (HIP) was negative only in one isolate (TPV34.08.05). From these results three different numerical profiles were obtained (5142110, 7140010 and 7143110) that were identified as belonging to *Enterococcus avium*, *Aerococcus viridans* 2 and *Lactococcus lactis* ssp. *lactis*, respectively. Each of the profiles 7140010 and 7143110 were originated by 48.5 % of the isolates, and the profile 5142110 was obtained in only one isolate (3 %). Isolates from three fish farms (NFM, TG and TC) originated the same numerical profile (7143110). The type strain and the Spanish isolate originate the same numerical profile (5140010) that was identified as *Leuconostoc* ssp. After 24 h of incubation we observed the following reaction alterations: all Portuguese strains became positive to RIB and MAN and originated variable results with LAC and AMD. However, in the case of LAC only one strain (TPV34.08.05) gave a positive result, with all others negative. From these results three numerical profiles were obtained (5042010, 7142110 and 7143110) that were identified as belonging to *L. lactis* ssp. *lactis* (5042010 and 7143110) and *E. avium* with possibility of *L. lactis* ssp. *lactis* (7142110). It was interesting to observe that all strains that originated the profile 7140010 after 4 h originated the profile 7142110 after 24 h of incubation, and the strains that exhibited the profile 7143110 after 4 h of incubation, maintained the same profile after 24 h. Consequently, isolates from farm NFM, TG and TC showed the same numerical profile (7143110) after 24 h (Table 12). The type strain and the Spanish isolate originated the same numerical profile (5142010) that was identified as

Table 12 - Date of isolation, number of strains and numerical profiles obtained with the API 20Strep and RAPID 32 Strep systems for the *Lactococcus garvieae* strains isolated in Portugal, after 24 h incubation.

Fish Farm	Date of isolation	Strains analyzed	API 20Strep	RAPID 32Strep
NFM	July, 2005	2	7143110	3033101131
PC	October, 2004	7	7142110	
	October, 2009	4	7143110	
TPV	July, 2004	3	7142110 7143110	3032311131 3033101131
	August, 2009	9	7142110 7143110 5042010	
	September, 2009	5	7142110 7143110	
TG	June, 2004	2	7143110	3033101131
TC	June, 2004	2		

Leuconostoc ssp..

With the RAPID 32Strep system, and after a 4 h incubation, none of the isolates was able to utilize β GAR, β GUR, α -galactosidase (α GAL), PAL, SOR, LAC, RAF, β GAL, N-acetyl- β -glucosaminidase (β NAG), glicil triptophane arylamidase (GTA), HIP, glycogen (GLYG), pululan (PUL), melibiose (MEL), melezitose (MLZ), L-arabinose (LARA), D-arabitol (DARL), α -cyclodextrine (CDEX) and urease (URE). All isolates were positive to ADH, β -glucosidase (β GLU), MAN, VP, alanine phenylalanine proline arylamidase (APPA), PyrA, maltose (MAL), and variable results were observed among strains for RIB, TRE, sucrose (SAC), methyl- β -D-glucopyranoside (M β DG), TAG, β -mannosidase (β MAN). With these results we obtained six different numerical profiles: five of them were identified as belonging to *L. garvieae* (30203101071, 30203101171, 30223101131, 30323101111, 30223101121), and one to *Enterococcus faecalis* (30333101171). The type strain and the Spanish strain were both identified as *L. garvieae*, even though the numerical profiles were, respectively, 30223101131 and 30223101120. With this system, after a 24 h incubation and when compared with the 4 h incubation period, tests RIB, TRE, SAC, CDEX, M β DG and TAG became positive, RAF, β MAN became negative, with LAC gave variable results. With these results two different numerical profiles (30333101131, 30323101131) were obtained and both were identified as *L. garvieae*, with 99 % percentage identity. The Spanish strain originated the profile 30333101131 (*L. garvieae*) and the type strain (30773101751) was identified as *E. faecalis*. Again, the isolates from farms NFM, TG and TC originated the same numerical profile (30333101131) (Table 12).

Using the PCR protocol and primers developed by Zlotkin *et al.* (1998) all the isolates used in this study gave the expected 1100 bp PCR amplification product specific for *L. garvieae*.

Using primers P5 and M13, we were able to type all *L. garvieae* strains used in this study, revealing a typeability of 100 %. With primer P5 the Portuguese strains of *L. garvieae* showed minor pattern differences among them, clustering together in a close genetic relationship and grouping in a single genogroup. These strains also clustered together with the Spanish strains PP62.1, showing that they belong to the genogroup A of Ravelo *et al.* (2001).

Using primer M13, a considerable number of bands (between 6 and 19) was generated, particularly in the range of 300 to 800 bp (Figure 14). With this technique 18 different DNA fragment profiles, designated RAPD types A to R (Figure 15) were established, with three big groups being observed (I, II and III). Group I contained the type and Spanish, as well as two strains from farm TPV from 2005 and one strain from farm PC from 2004 (Figure 15). Group II contained most of the strains from farm TPV from 2005 and from farm PC from 2004 and 2005. Finally, Group III contained all

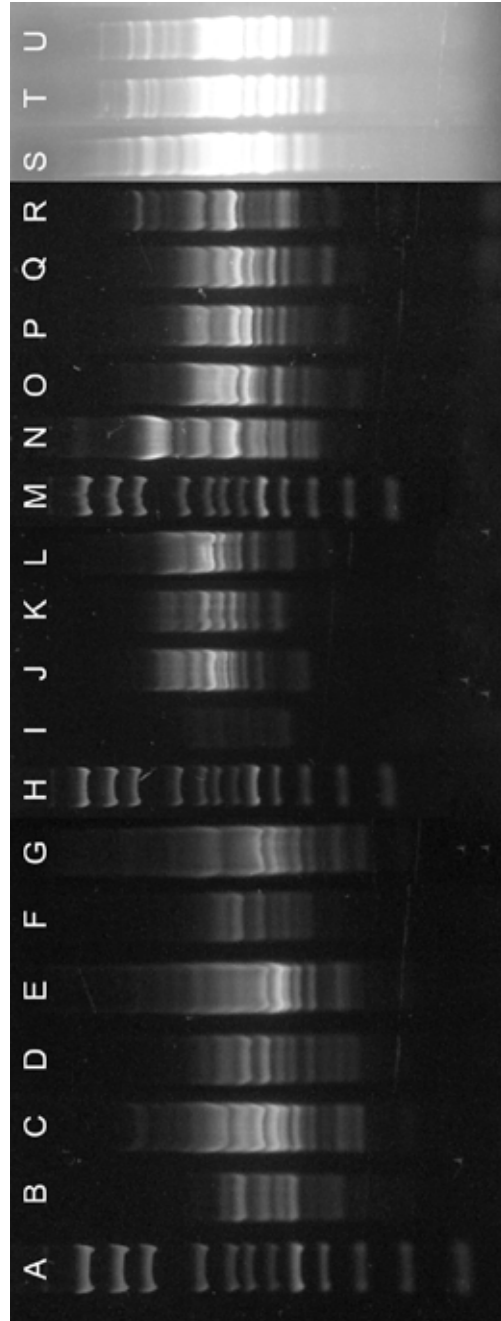


Figure 14 - The 18 different DNA fragment profiles, obtained in *Lactococcus garvieae* when M13 was applied for RAPD analysis. Lane A, H and M - 100 bp Molecular weight ladder (Solis BioDyne, Estonia); Lane B - RAPD type P; Lane C - RAPD type I; Lane D - RAPD type O; Lane E - RAPD type D; Lane F - RAPD type H; Lane G - RAPD type F; Lane I - RAPD type M; Lane J - RAPD type R; Lane K - RAPD type N; Lane L - RAPD type L; Lane N - RAPD type E; Lane O - RAPD type K; Lane P - RAPD type J; Lane Q - RAPD type G; Lane R - RAPD type Q; Lane S - RAPD type C; Lane T - RAPD type A; Lane U - RAPD type B.

strains from farms TC and TG and the rest of the strains from farm PC from 2004 and from farm TPV from 2004. Between groups we could observe a big difference in the number of bands (Group I - 18-19 bands; Group II - 9-12 bands; Group III - 6-14 bands) (Figure 15). Of the fourteen strains isolated in farm TPV during 2005 only two did not group together in RAPD types (TPV19.09.05 and TPV25.09.05), however TPV19.09.05 joined together with the two main RAPD types of the TPV strains isolated in 2005 at a distance of 0.325. Two of the three strains isolated from farm TPV during 2004 also constituted a RAPD type (Figure 15). And all of the strains from TPV isolated in 2004 belong to group III, and the strains from 2005 belong to groups I and II, however in group I we could only find strains from August 2005.

The two strains from farm TG originated a single RAPD type as did the two strains from farm TC. Most of the strains from farm PC were included in three different RAPD types, one had three of the four strains from 2009, and the other 2 had most of the strains from 2004. However, the strains from 2004 were present in the three main groups (Group I, II and III), and this was the only fish farm where this happened (Figure 15). In only one RAPD type we could verify the presence of strains from more than one fish farm (the two strains from farm TG and one strain from farm TPV from 2004) (Figure 15).

The application of the REP-PCR technique with the primer (GTG)₅ to the Portuguese isolates of *L. garvieae* allowed the typing of all the isolates (100 % typeability), generating a considerable number of bands (between 14 and 18, with most strains having 16 bands) particularly in the range from 100 to 3000 bp (Figure 16). This was the typing technique that originated the biggest number of bands per strain. With this technique 11 different DNA fragment profiles were established (REP types A to K) (Figure 16).

With the application of this typing technique and with some exceptions, all strains isolated from the same fish farm on the same year presented the same banding pattern and were grouped on a single REP type. The biggest exception were the two isolates from farm NFM (Figure 17).

Also with the exception of TPV34.08.05, all other strains from farm TPV isolated during 2005 grouped together in a single REP type (FIGURE 17), as did the strains from 2004. However the REP type composed of strains isolated in 2005 clustered first with the type, Spanish and TG and then with the REP types composed of strains from farms TG and PC (2009) and then with the REP type composed of the strains from the same farm isolated in 2004.

The application of the BOX-PCR typing technique to the Portuguese strains of *L. garvieae* allowed the typing of all isolates (100 % typeability). This was the typing technique that originated the lowest number of bands (between 3 and 13 bands, with

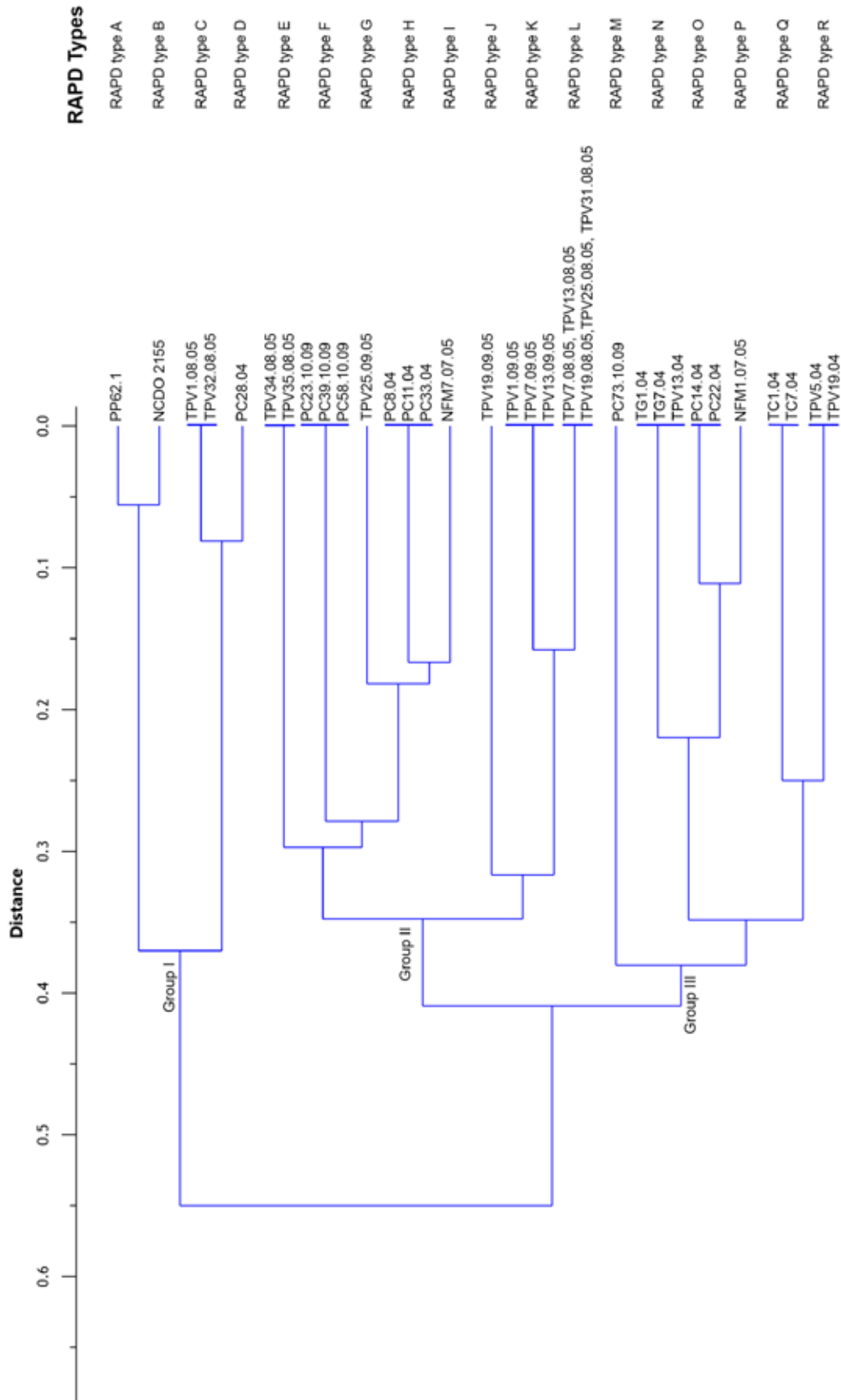


Figure 15 - Dendrogram established by Phoretix1D Pro software package (TotalLab, UK) using the Dice similarity coefficient and UPGMA on the basis of the RAPD profiles obtained with *Lactococcus garvieae* when using primer M13.

most of them originating 7 bands) (Figure 18). With this technique 12 different DNA fragment profiles were established (BOX types A to L) (Figure 19).

With this typing technique, and with the exception of strain PC33.04, all strains from farm PC, independently of year of isolation, were grouped on a single BOX type. For farm TPV, where strains from different years were obtained, the same situation did not occur; for each year of isolation two different BOX types were obtained, even though most of the strains isolated in 2005 were grouped in one BOX type (BOX type I, Figure 19). Strains from the farm NFM were grouped in one BOX type, as were the strains from farm TG.

When considering a similarity distance of 0.45, three different groups could be established (Group I, II and III) (Figure 20). Group I, included the Spanish strain; Group II included all but one Portuguese strains and Group III included the type and the remaining Portuguese strain.

Using the PFGE technique, the *Sma*I enzyme generated nine different DNA fragment profiles, designated pulsotypes A to I, with 12 to 21 bands over a size ranging of about 20.5 to 360 kb (Figure 20). Figure 21 shows the dendrogram obtained with the nine different pulsotypes after UPGMA clustering. As we can observe, 75.6 % of the Portuguese strains of *L. garvieae* belong to one single pulsotype (G), independently of the year or place of isolation. The only exceptions are the isolates from farm TC that form one single pulsotype (A), and the strains from farm PC from 2004, that are distributed in three different pulsotypes (E, F and I), with only one pulsotype containing more than one strain from this farm (pulsotype E). The Spanish strain was closely related to the Portuguese isolates of pulsotype I (differing by five restriction fragments) and to a lesser extent to the ones from the major pulsotype G (differing by four restriction fragments). The type strain (NCDO 2155) constituted its own pulsotype (C). The other two pulsotypes (B and D) contained only one strain each, from farm TPV, but isolated in different years.

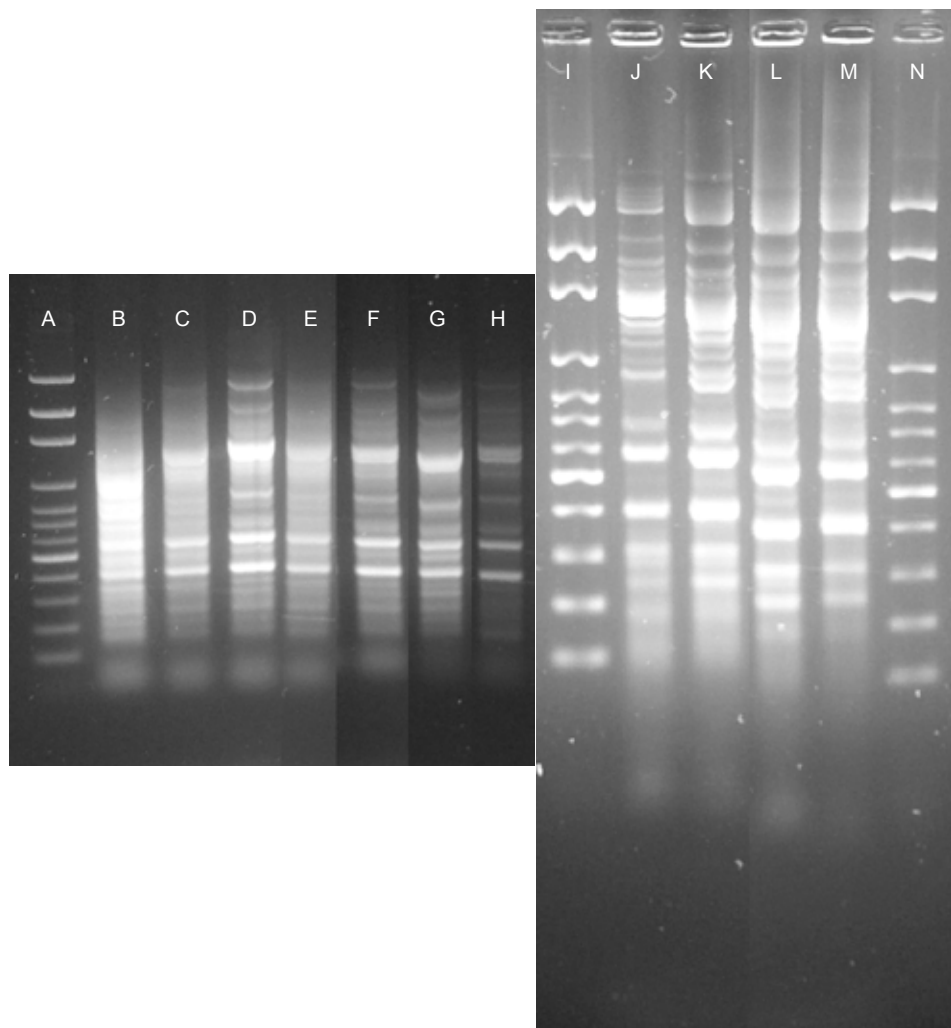


Figure 16 - The 11 different DNA fragment profiles, obtained with *Lactococcus garvieae* when the REP primer (GTG)₅ was applied. Lane A, I and N - 100 bp Molecular weight ladder (Solis BioDyne, Estonia); Lane B - REP type C; Lane C - REP type B; Lane D - REP type G; Lane E - REP type E; Lane F - REP type A; Lane G - REP type H; Lane H - REP type F; Lane J - REP type D; Lane K - REP type J; Lane L - REP type K; Lane M - REP type I.

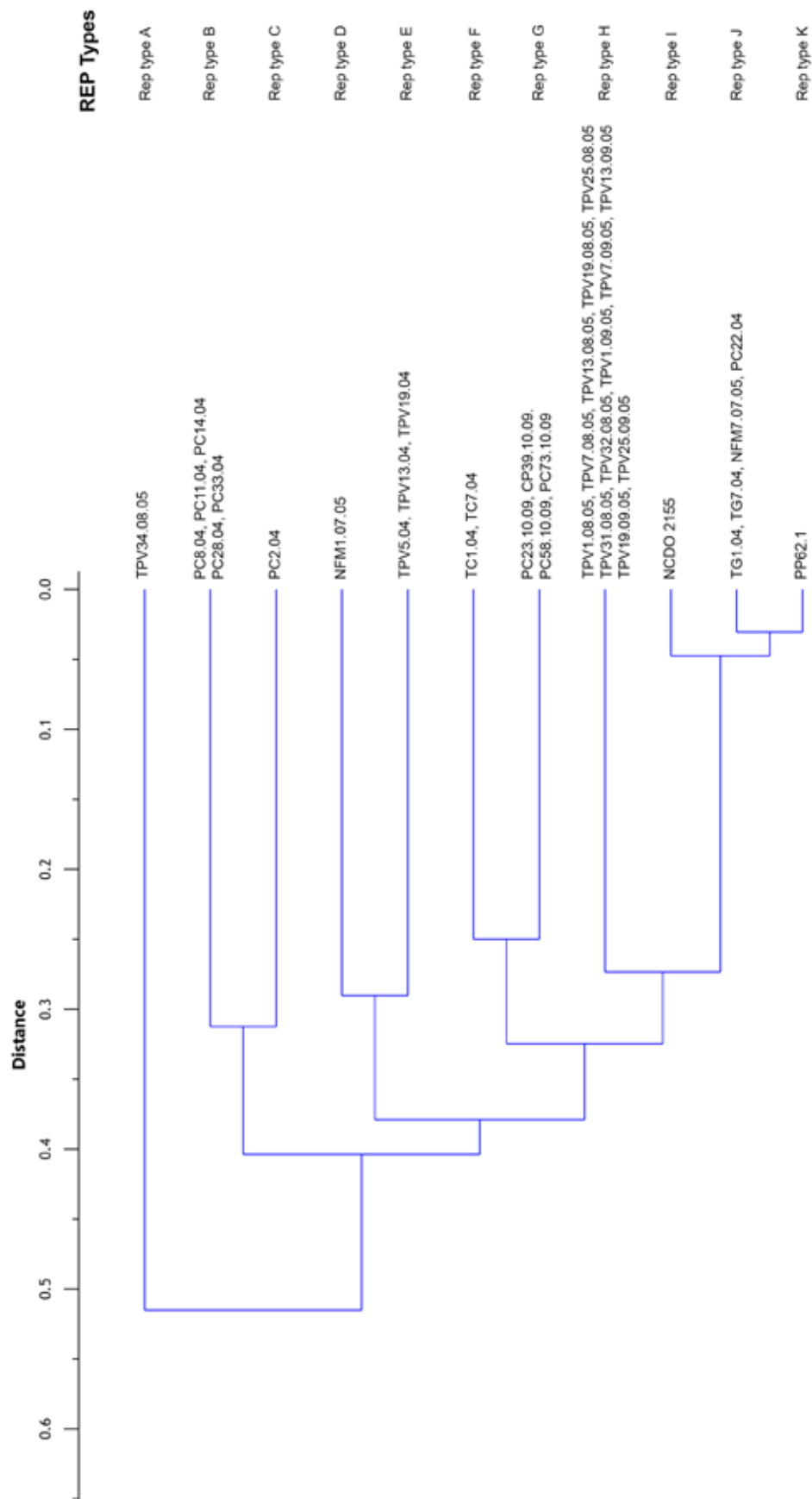


Figure 17 - Dendrogram established by Phoretix1D Pro software package (TotalLab, UK) using the Dice similarity coefficient and UPGMA on the basis of the REP-PCR profiles of *Lactococcus garvieae* obtained with primer (GTG)₅.

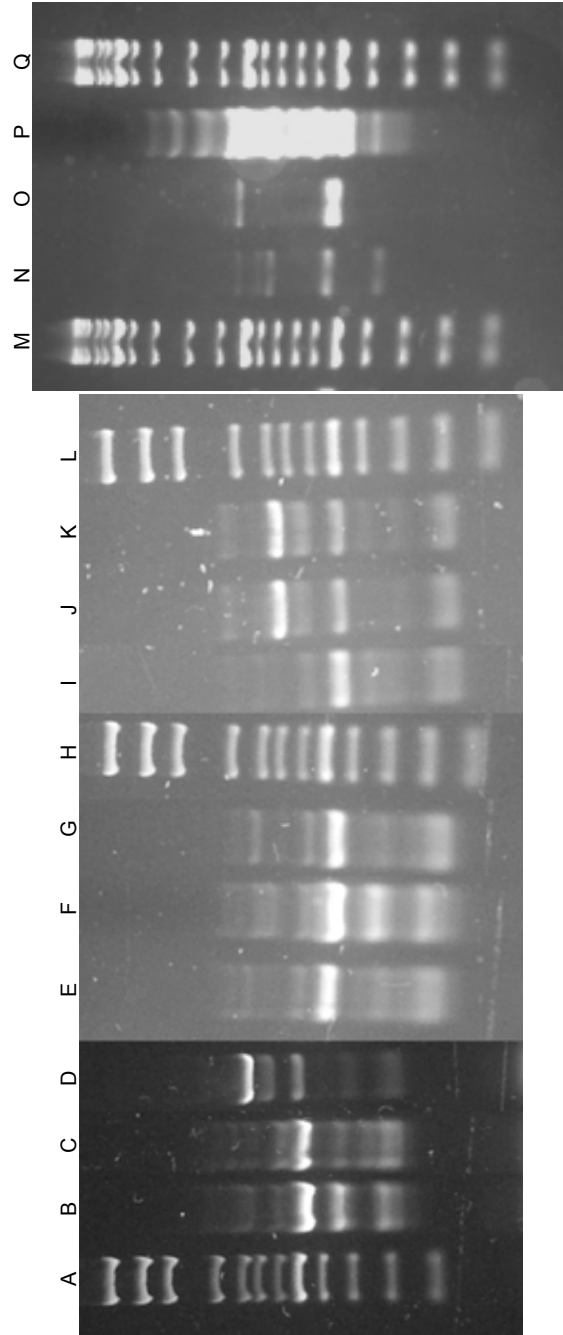


Figure 18 - The 12 different DNA fragment profiles obtained with *Lactococcus garvieae* when primer BOXA1-R was applied. Lane A, H, L, M and Q Molecular weight ladder; Lane B - BOX type H; Lane C - BOX type E; Lane D - BOX type J; Lane E - BOX type D; Lane F - BOX type I; Lane G - BOX type C; Lane I - BOX type F; Lane J - BOX type G; Lane K - BOX type B; Lane N - BOX type K; Lane O - BOX type A; Lane P - BOX type L.

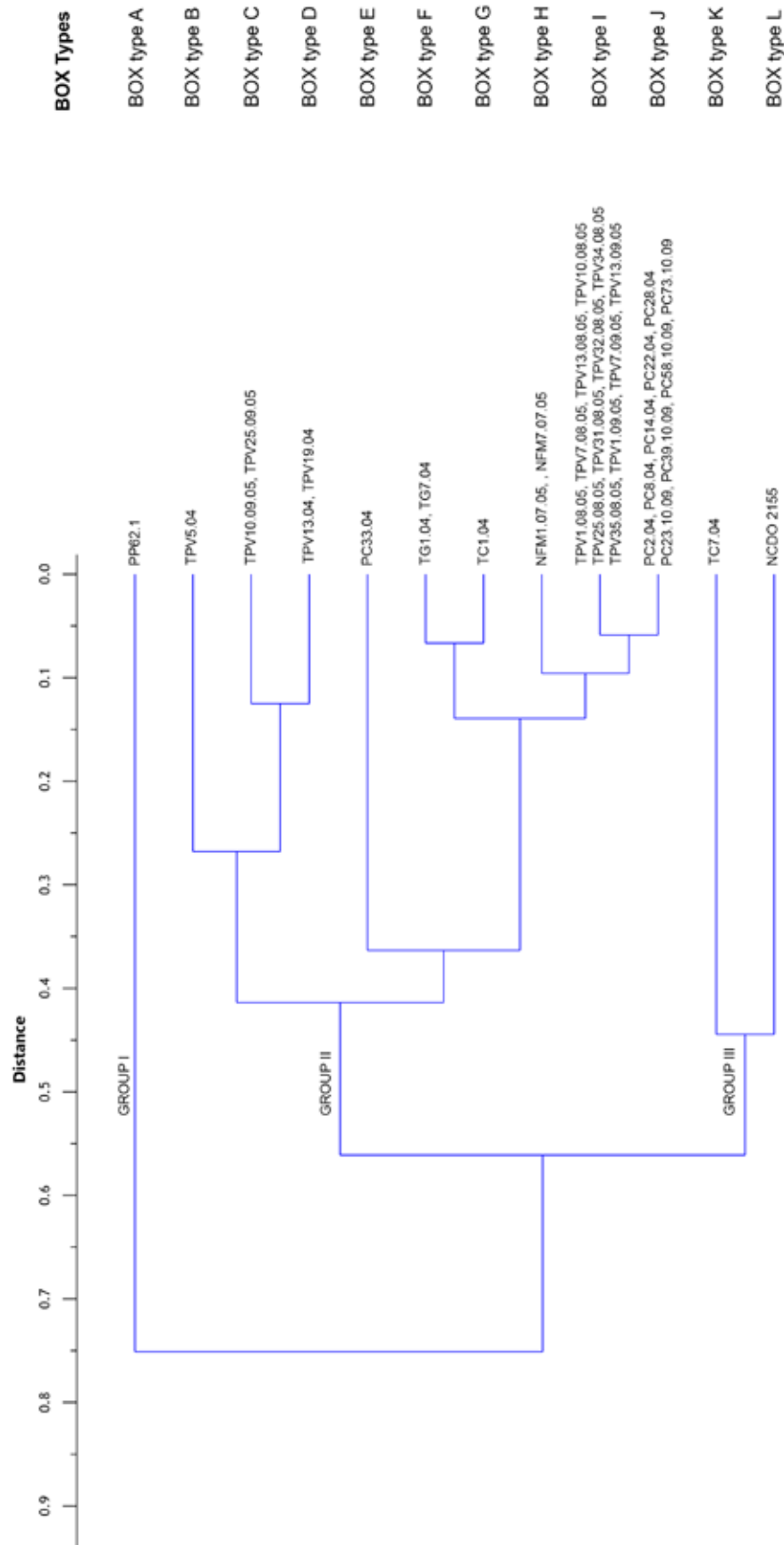


Figure 19 - Dendrogram established by Phoretix 1D Pro software package (TotalLab, UK) using the Dice similarity coefficient and UPGMA on the basis of the BOX elements profiles obtained with *Lactococcus garvieae* strains analysed and obtained with primer BOXA1-R was applied.

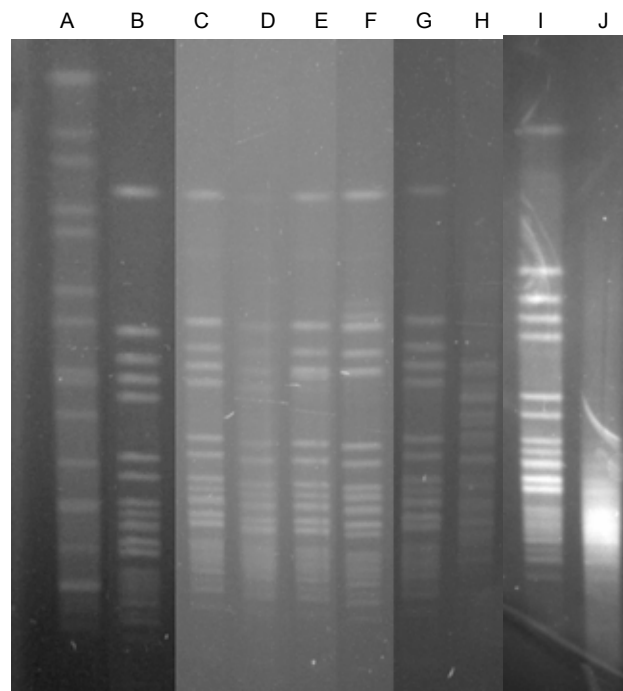


Figure 20 - The nine different pulsotypes obtained with digestion of *Lactococcus garvieae* DNA with the restriction enzyme *Sma*I. Lane A - Molecular size marker (*Xba*I digestion of the DNA of *Salmonella* Braenderup); Lane B - Pulsotype G type H; Lane C - Pulsotype D; Lane D - Pulsotype B ; Lane E - Pulsotype E; Lane F - Pulsotype F; Lane G - Pulsotype I; Lane I - Pulsotype A; Lane J - Pulsotype H; Lane K - Pulsotype C.

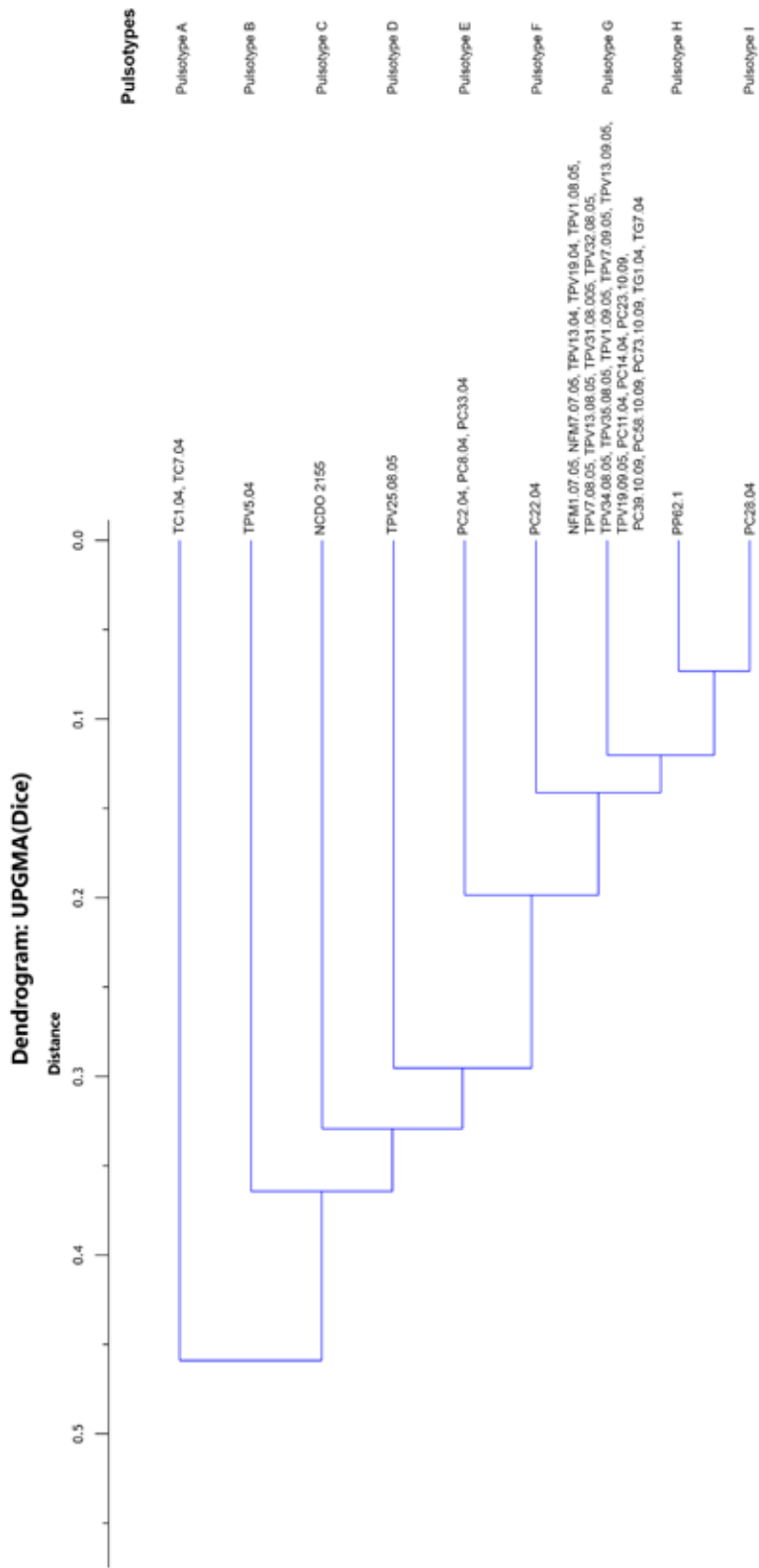


Figure 21 - Dendrogram of *Lactococcus garvieae* isolates established by Phoretix1D Pro software (TotalLab, UK) based on the Dice similarity coefficient and UPGMA cluster analysis of the nine different *Apal* pulsotypes.

Characterization of Portuguese strains of *Streptococcus parauberis*

Streptococcus parauberis occurred throughout the sampling period with severe outbreaks in May, July and August 2004, when clinical signs were more severe and mortality higher (cumulative mortality up to 10 %). Some fish showed poor appetite and abnormal swimming behavior with sharp and sudden erratic movements. Moribund fish were emaciated, with uni- or bilateral exophthalmia with abscesses and haemorrhages (Fig. 22A, B). Accumulation of fluid in the dorsal region (Fig. 22A) and at the base of the fins, haemorrhage in the dorsal and caudal fins, occasionally around the mouth and abdominal petechia. Internal signs included pale, friable liver, occasionally hepatomegaly, haemorrhagic and friable kidney, and ascitic fluid in the peritoneal cavity.

Growth was observed on TSA-1 and BA medium from all examined organs. No colonies were recovered on TCBS agar. Pinpoint colonies 1-2 mm in diameter, raised, grayish-white with entire margin and α -haemolysis producers were detected in BA after 24-48 h incubation at 22° C. In TSA-1 pinpoint colonies with entire margin, 1-3 mm in diameter, raised and white were observed.

The histological analysis of the different organs of turbot showed that *Streptococcus parauberis* induced subacute to chronic inflammations. The main histopathological change observed was hyperplasia of the meninges.

In the skin, small chains of Gram-positive bacteria could be observed in the dermal stratum spongiosum (Figure 23A), that in some areas was necrotic. Also, the muscle beneath the injured skin showed necrosis (Figure 23A).

In the brain, bacterial cells could be observed in the meninges (Fig. 23B). Hyperplasia, hydropic degeneration and large amounts of bacteria were detected in the dura-mater of the brain (Figure 23B, C) with an associated infiltration of leucocytes,

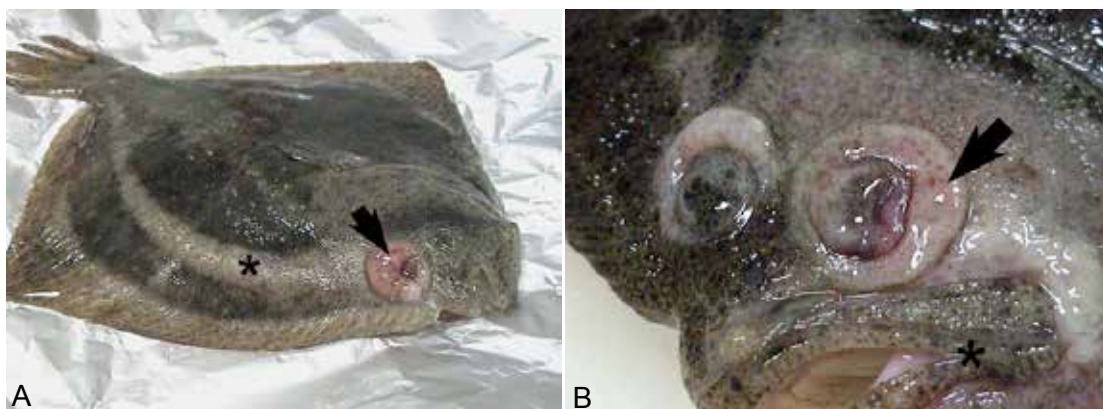


Figure 22 - Macroscopic lesions associated with *Streptococcus parauberis* infection in turbot. (A) Unilateral exophthalmia and eye hemorrhage (arrow), and accumulation of fluid at the dorsal region (*) scale bar = 2 cm. (B) Exophthalmia and periorbital abscesses. Hemorrhage in the eyes (arrows) and mouth (*) scale bar = 1 cm.

with a high number of bacteria present inside mononuclear cells (Figure 23D, E). In heavily infected meninges necrotic tissue was also observed. All bacteria present in the brain were Gram-positive.

In the eyes of heavily infected fish, different degrees of inflammation associated with hypertrophy and necrosis of choroid connective tissue could be observed. Mononuclear inflammatory cells with bacteria could also be observed (Figure 23F).

In the liver, Gram-positive bacteria were observed only in the portal space connective tissue (Figure 23G). However, in some areas necrotic tissue was found. In the kidney, Gram-positive cocci were only observed in the supporting connective tissue. Also, some areas of the kidney showed necrotic areas.

All strains exhibited the same phenotypic pattern: non-motile, Gram-positive cocci grouped in pairs or in short chains, facultative anaerobic, oxidase and catalase negative, producing acid from glucose, mannitol, sorbitol and lactose, but not from arabinose and melibiose. Arginine dihydrolase, lysine and ornithine decarboxylase, H₂S, indol and MR were negatives, and Voges-Proskauer was positive. All strains were able to grow from 4 °C to 40 °C and at 1 % NaCl but not at 4.5 and 6.5 % (Table 13).

In the API 20Strep system, with a 4 h incubation period, all strains tested were PYRA, αGAL, βGUR, βGAL, ADH, RIB, ARA, MAN, SOR, LAC, INU, RAF, AMD and GLYG negative. VP, HIP, ESC, PAL and LAP were positive. TRE was positive in only two isolates and negative in all other isolates. Consequently, all the isolates were distributed within two identifiable numerical profiles (7060000 and 7060010). According to the API database, the profiles obtained correspond to a low discriminative identification (between *Aerococcus urinae* and *Lactococcus lactis* ssp. *cremosis*) and a doubtful profile (between *Listeria* sp., *A. urinae*, *L. lactis* ssp. *cremoris* and *Streptococcus constellatus*) respectively. The profile 7060000 was the most usual one since it was obtained in 92.3 % of the isolates. The type strain and the South Korean strains originated the same numerical profile 7060000. After 24 h incubation, with exception of RIB, MAN, LAC and RAF (that showed variable results) all tests gave the same results as after 4 h incubation. From these results, two different numerical profiles were again obtained (7060010 and 7060110). The profile 7060010 was obtained in 15.4 % of the strains, and it resulted from isolates that previously originated the 7060000 profile. According to the API database, the profile 7060110 corresponded to an acceptable identification with *A. urinae* as a significant taxa and *L. lactis* ssp. *cremosis* as the next taxon. The type strain and the two South Korean strains originated three different numerical profiles (7060410, 7060510, 7062550) that corresponded to a low discriminative identification. Two of them (7060410 and 7060510) had *L. lactis* ssp. *cremosis* and *A. urinae* as significant taxa, and the other one (7062550) had *L. lactis* ssp. *cremosis* and *Enterococcus avium* as significant taxa.

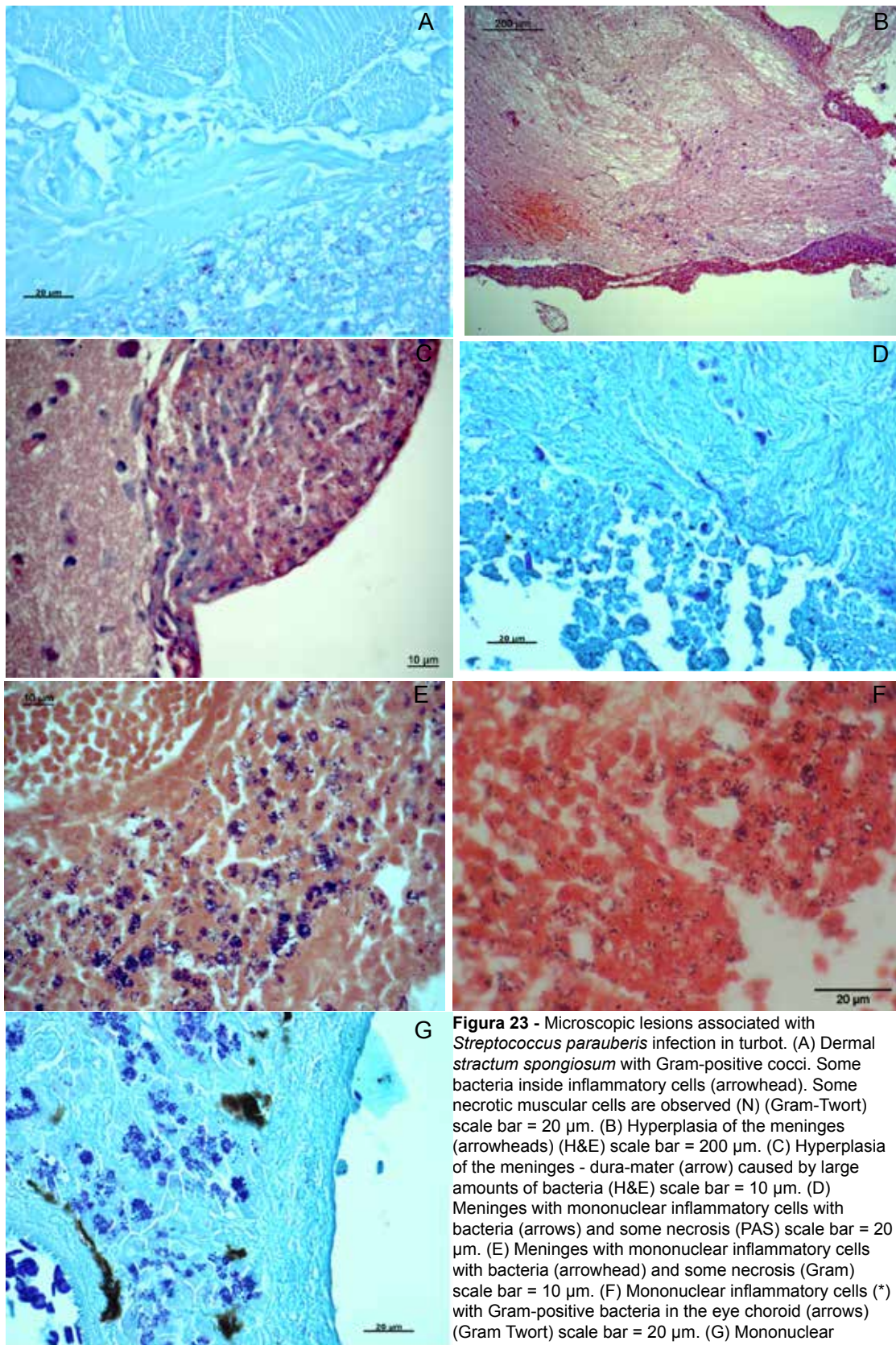


Figura 23 - Microscopic lesions associated with *Streptococcus parauberis* infection in turbot. (A) Dermal stratum spongiosum with Gram-positive cocci. Some bacteria inside inflammatory cells (arrowhead). Some necrotic muscular cells are observed (N) (Gram-Twort) scale bar = 20 μ m. (B) Hyperplasia of the meninges (arrowheads) (H&E) scale bar = 200 μ m. (C) Hyperplasia of the meninges - dura-mater (arrow) caused by large amounts of bacteria (H&E) scale bar = 10 μ m. (D) Meninges with mononuclear inflammatory cells with bacteria (arrows) and some necrosis (PAS) scale bar = 20 μ m. (E) Meninges with mononuclear inflammatory cells with bacteria (arrowhead) and some necrosis (Gram) scale bar = 10 μ m. (F) Mononuclear inflammatory cells (*) with Gram-positive bacteria in the eye choroid (arrows) (Gram Twort) scale bar = 20 μ m. (G) Mononuclear inflammatory cells with Gram-positive bacteria in the liver

portal space (arrows), but not inside de blood vessels (Gram Twort) scale bar = 20 μ m (adapted from Ramos et al., 2012).

Table 13 - Phenotypic characteristics of *Streptococcus parauberis* used in this study

Character	Strains		
	Portuguese strain (n=32)	South Korean strain (n=1)	Type strain NCDO 2020
Gram stain	+	+	+
Motility	-	-	-
Oxidase	-	-	-
Catalase	-	-	-
Pigment production	-	-	-
Haemolytic type	α	α	α
ZOF	F	F	F
Indole	-	-	-
H ₂ S production	-	-	-
Voges-Proskauer	+	+	+
Methyl-Red	-	-	-
Hydrolysis of:			
Arginine	-	-	-
Decarboxylation of:			
Lysine	-	-	-
Ornithine	-	-	-
Growth at:			
4 °C	+	+	+
10 °C	+	+	+
25 °C	+	+	+
40 °C	+	+	+
Growth in:			
1 % NaCl	+	+	+
4.5 % NaCl	-	-	-
6.5 % NaCl	-	-	-
Acid from:			
Arabinose	-	-	-
Lactose	+	+	+
Mannitol	+	+	+
Melibiose	-	-	-
Sorbitol	+	+	+

Using the RAPID 32Strep system, and after a 4 h incubation, none of the isolates were able to utilize β GAR, β GUR, α GAL, PAL, SOR, RAF, β GAL, PYRA, GTA, GLYG, PUL, MEL, MLZ, LARA, DARL, TAG, β MAN and URE. All isolates were positive to β GLU, APPA and HIP, and variable results were observed among strains for ADH, RIB, MAN, LAC, TRE, VP, β NAG, MAL, SAC, M β DG and CDEX. With these results, all the isolates were distributed within eleven identifiable numerical profiles (20003010000, 20022010011, 20022010100, 20023010000, 20023010100, 20023011110, 30022010000, 30022010110, 30022011100, 30022011110, 30032210110).

These profiles corresponded to an excellent (20003010000) and a very good identification (20023010000) of *L. lactis* ssp. *cremosis*; to an excellent identification of *A. viridans* (20023011110); to a good identification *L. lactis* ssp. *lactis* (30022011110). With doubtful profiles we had profiles 20022010011 (with *L. lactis* ssp. *lactis* as a significant taxa), 20023010100 (between *Streptococcus equinus*, *Leuconostoc* spp., *L. lactis* ssp. *cremoris* and *S. alactolyticus*), 30022010000 (between *L. lactis* ssp. *lactis*, *L. lactis* ssp. *cremoris* and *L. garvieae*), 30022010110 (between *L. lactis* ssp. *lactis*, and *L. garvieae*), 30022011100 (between *L. lactis* ssp. *lactis* and *S. sanguinis* 2). With an unacceptable profile we had the profile 30022210100 (with *L. lactis* ssp. *lactis* as a significant taxa), and with a not valid identification we had the profile 20022010100 (between *S. mitis* 2 and *A. viridans*). The type strain originated the profile 20032010100 that in the API database corresponded to a doubtful profile (between *S. mitis* 2, *L. lactis* ssp. *cremoris* and *A. viridans*), and the two Korean isolates originated two different numerical profiles (30032011110 and 30332011100) that in the API database corresponded to an acceptable identification of *L. lactis* ssp. *lactis*.

After a 24 h incubation, we observed that when compared with 4 h incubation, tests RIB, MAN, TRE, MAL, M β DG, SAC, β MAN became positive, and β GUR, PAL, SOR, RAF, β GUR, TAG, LARA, DARL, TAG gave variable results. All other tests gave the same results as after 4 h incubation. With these results we obtained eight different identifiable numerical profiles (30323011150, 20723011150, 30323011150, 30723011150, 34322011150, 34323011150, 34722011150, 34723011150, 34752011150) that in the API database corresponded to doubtful profiles. However, five of them (20723011150, 30723011150, 34722011150, 34723011150, 34752011150) identified the isolates as *S. uberis* 2 with a percentage of ID higher than 99,0 %. The other profiles, also identified the isolates as *S. uberis* 2, but at a lower percentage of ID, and not always as the first species: 30323011150 - *L. lactis* ssp. *lactis* (76.7 % ID) and *S. uberis* 2 (23.2 % ID); 34322011150 - *L. lactis* ssp. *lactis* (86,6 % ID), *S. uberis* 2 (8.1 % ID) and *S. anguinosus* (5 %); 34323011150 - *S. uberis* 2 (68,5 % ID), *L. lactis* ssp. *lactis* (20.4 % ID) and *S. anguinosus* (10.5 % ID). The type strain originated the profile 30772001751, which in the API database is an unacceptable profile, but identifies the isolate as *S. uberis* 2. The two South Korean isolates originated two different identifiable numerical profiles (34732011150 and 35772011170) that in the API database corresponded to doubtful profiles, but that identified the isolates as *S. uberis* 2 with a percentage of ID of 99.5 % and 84.1 %.

In the hemolysis test, all Portuguese isolates were α -haemolytic. By slide agglutination test all *S. parauberis* gave a positive and strong reaction.

Using the PCR protocol and primers developed by Hassan *et al.* (2001) all the isolates used in this study gave the expected 884 bp PCR amplification product specific for *S. parauberis*.

The susceptibility pattern of the bacterial isolates to the 11 antimicrobial drugs tested is shown in Table 14. All Portuguese strains were sensitive to erythromycin, chloramphenicol, nitrofurantoin, trimethoprim-sulfamethoxazole and penicillin, resistant to tetracycline and showed a different degree of sensibility to the other chemical compounds used (Table 14).

Table 14 - Susceptibility of *Streptococcus parauberis* isolated in Portugal to antimicrobial agents. Between brackets are shown the number of isolates that gave that result.

Resistance/Sensitivity to:	Isolates	
	Portuguese (n=32)	Type Strain (NCDO 2020)
Penicilin G (10)	S	R
Ampicilin (10)	S (26)	S
Erythromycin (10)	S	R
Trimethoprim-sulfametazol (25)	S	R
Cefalotin (30)	S (20)	R
Chloramphenicol (30)	S	R
Nitrofurantoin (300)	S	R
Furazolidon (500)	R (27)	R
Tetracycline (30)	R	R
Streptomycin (10)	R (26)	R

The *tet(K)*, *tet(L)*, *tet(M)*, *tet(O)* and *tet(M/O/S)* genes were not detected in strains of *S. parauberis* isolated in Portugal. Only *tet(S)* was detected in some of the Portuguese strains of *S. parauberis* (CM333.04, CM461.04, CM468.04, CM1.01.05, CM21.01.05 and CM42.01.05) and in the type and South Korean strains.

Using the RAPD technique with primer M13 we were able to type all *S. parauberis* strains used in this study revealing a typeability of 100 %. This technique originated between 7 and 13 bands in the different fingerprints, particularly in the range of 300 to 1000 bp, but no common bands could be observed between the different fingerprints (Figure 24). Ten different RAPD types could be established (RAPD types A to J), but most of the Portuguese strains (80.7 %) were clustered in two main RAPD types (Figure 25) (RAPD type C and H). However these two main RAPD types could not be related to the time of isolation, since most of the strains were isolated at different time points. The exception were the strains isolated in September 2004 and most of the ones isolated in January 2005, that belonged to the same RAPD type (RAPD type C). Also, the type and the South Korean strains belonged to their own RAPD type (RAPD type A, B and F), and were distributed all over the dendogram. Most of the strains used in this study were isolated from the kidney of the fish and some from the brain, spleen

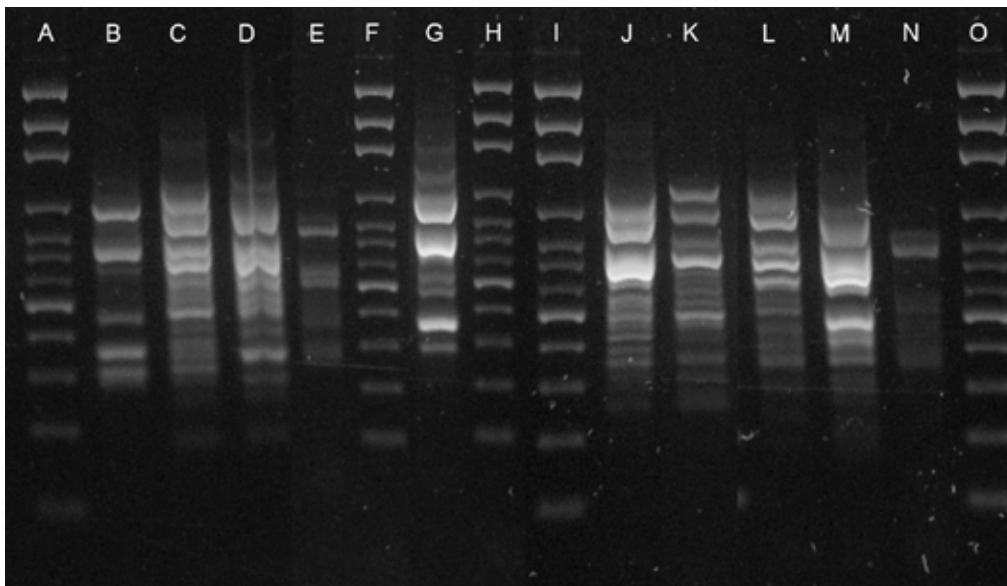


Figure 24 - The 10 different DNA fragment profiles obtained with *Streptococcus parauberis* strains when subjected to RAPD analysis with primer M13. Lane A, F, H, I, O - 100 bp Molecular weight ladder (Solis BioDyne, Estonia); Lane B - RAPD type D; Lane C - RAPD type G; Lane D - RAPD type F; Lane E - RAPD type H; Lane G - RAPD type B; Lane J - RAPD type E; Lane K - RAPD type J; Lane L - RAPD type C; Lane M - RAPD type I; Lane N - RAPD type A.

and eye, however no differences related to the organ of isolation could be found using the M13 primer.

The application of the REP-PCR technique with the primer (GTG)₅ to Portuguese isolates of *S. parauberis* revealed a typeability of 100 %. We were able to obtain between 11 and 16 bands in the different fingerprints, particularly in the range of 300 to 3000 bp, but no common bands could be observed between the different fingerprints (Figure 26). With this technique 10 different DNA fragment profiles were established (REP types A to J) (Figure 26).

With this technique we could not observe any relation between sampling dates or organ of isolation. However, isolates from brain and eye (CM47.04 and CM340.04, respectively) clustered together with two isolates from the kidney (REP type D). Also, all strains isolated in January 2005, had the same fingerprint (REP type H). The type strain and the South Korean strains belong to the same REP type (REP type A) (Figure 27).

The application of the BOX-PCR typing technique to the Portuguese isolates of *S. parauberis* revealed a typeability of 100 %. We were able to obtain between 4 and 18 bands in the different fingerprints, particularly in the range of 500 to 750 bp, with 24 different DNA fragment profiles being established and designated BOX types A to X (data not shown). Using this technique only two BOX types had more than one strain; one of them was formed by the South Korean strains, and the other by five of the Portuguese strains (Figure 28). No relationship between sampling dates or organ

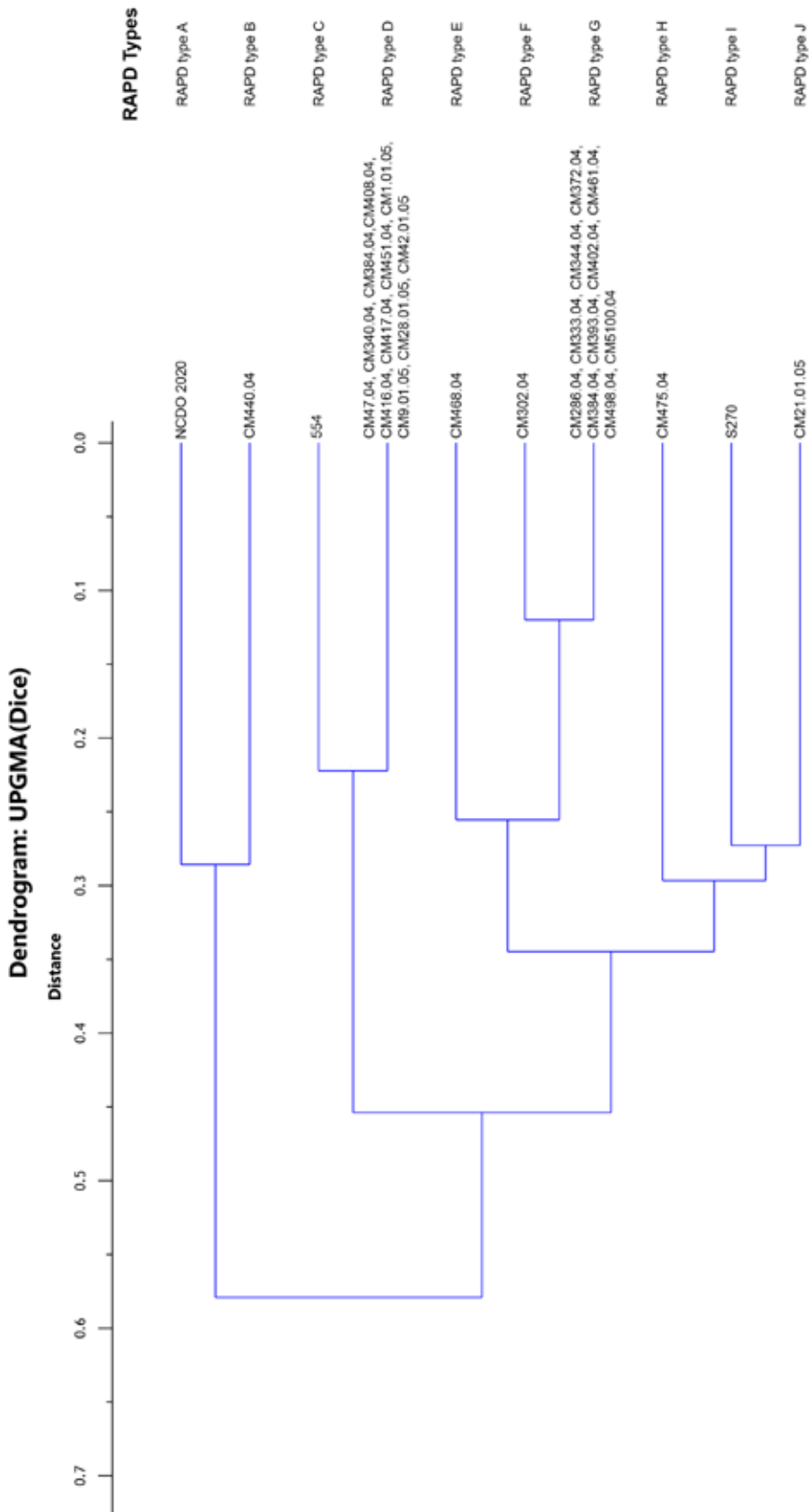


Figure 25 - Dendrogram established by Phoretix1D Pro software package (TotalLab, UK) using the Dice similarity coefficient and UPGMA obtained with *Streptococcus parauberis* strains when subjected to RAPD analysis with primer M13.

of isolation could be established.

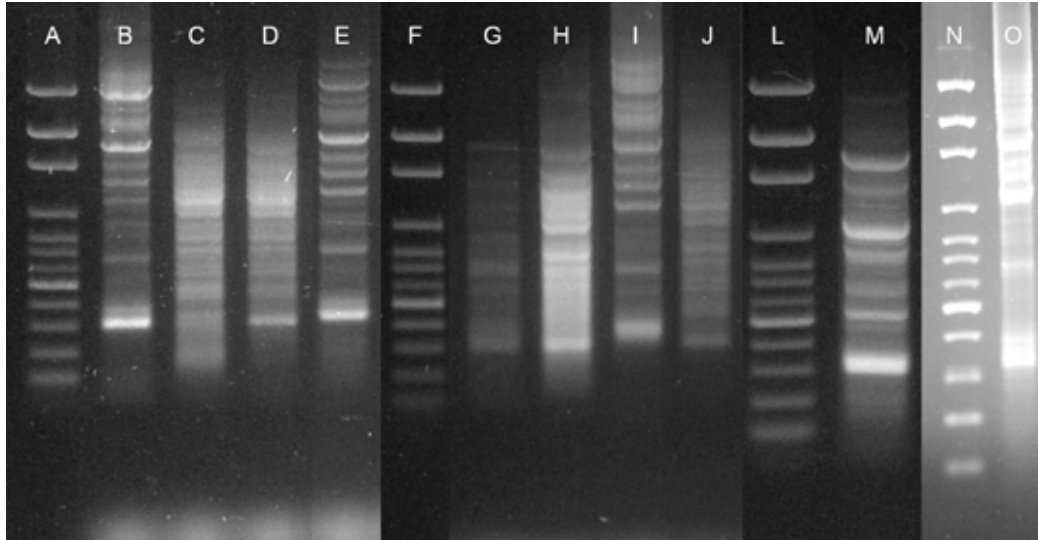


Figure 26 - The 10 different DNA fragment profiles obtained with *Streptococcus parauberis* strains when subjected to REP-PCR analysis with primer (GTG)₅. Lane A, F, L, O - 100 bp Molecular weight ladder (Solis BioDyne, Estonia); Lane B - REP type D; Lane C - REP type J; Lane D - REP type I; Lane E - REP type E; Lane G - REP type G; Lane J - REP type F; Lane K - REP type C; Lane L - REP type H; Lane M - REP type A; Lane N - REP type B.

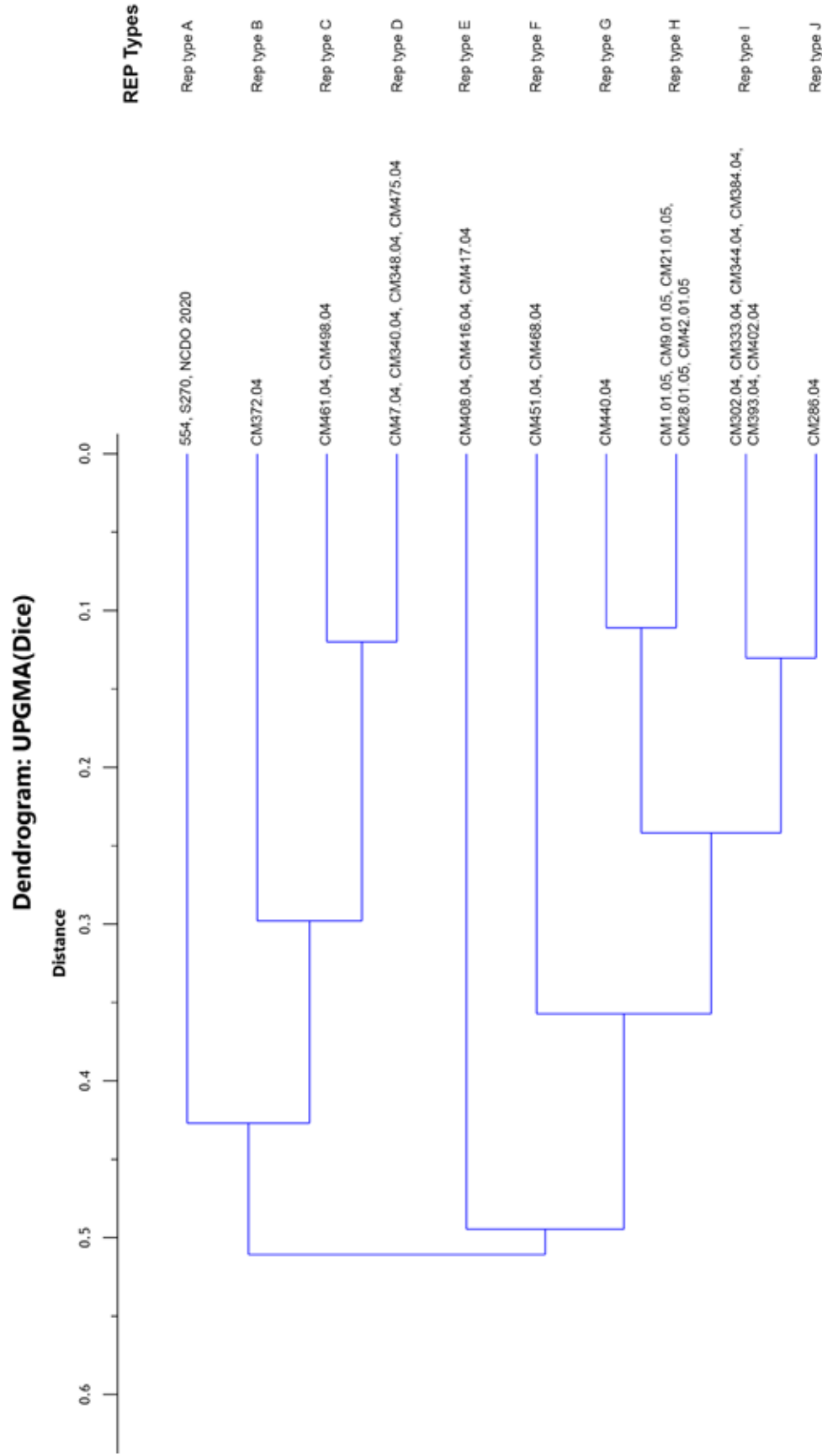


Figure 27 - Dendrogram established by Phoretix1D Pro software package (TotalLab, UK) using the Dice similarity coefficient and UPGMA on the basis of the REP-PCR profiles Obtained with *Streptococcus parauberis* when using primer (GTG)₅.

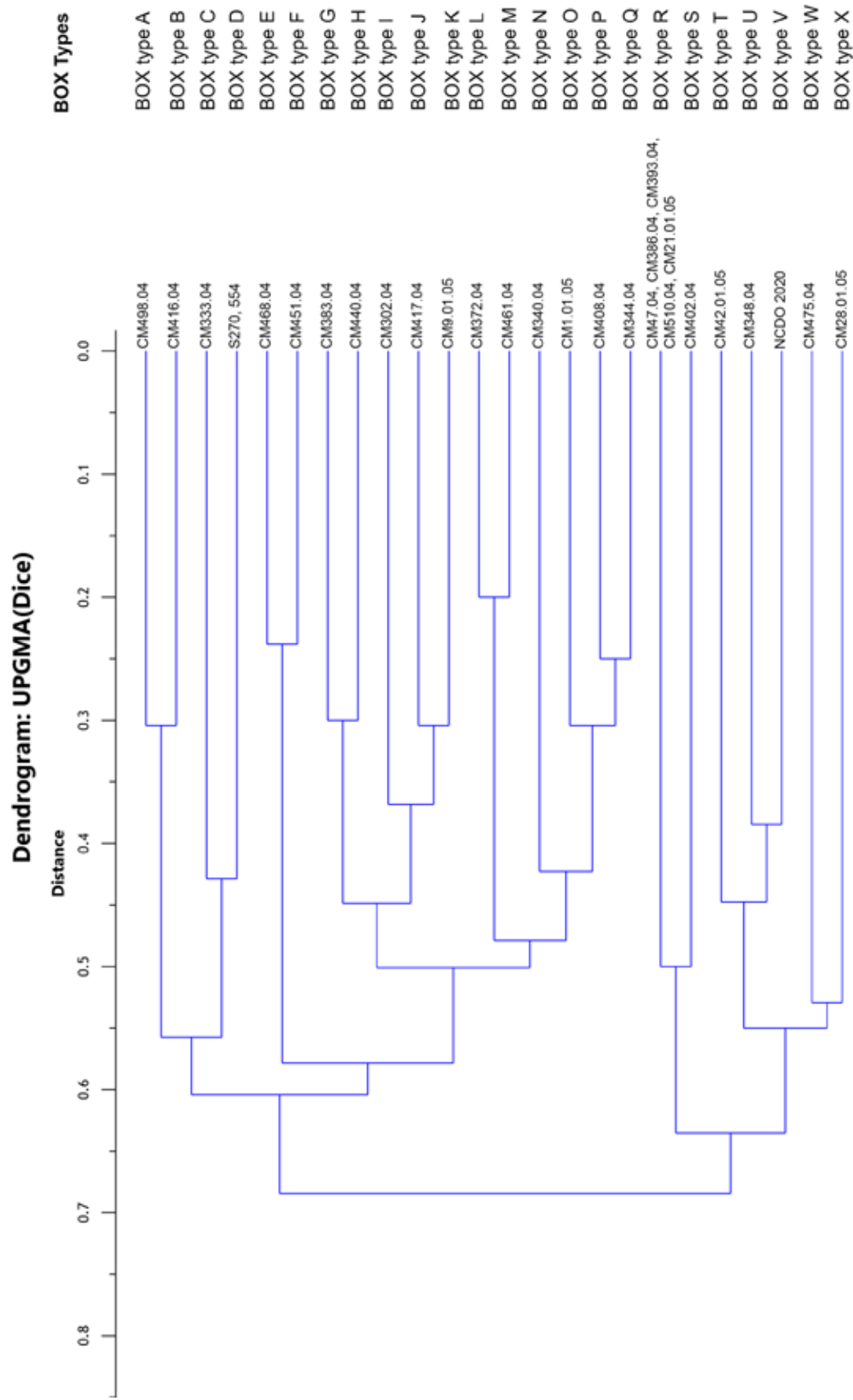


Figure 28 - Dendrogram established by Phoretix 1D Pro software package (TotalLab, UK) using the Dice similarity coefficient and UPGMA on the basis of the BOX elements profiles obtained with *Streptococcus parauberis* when using primer BOXA1-R.

Phages for treatment of *L. garvieae* and *A. salmonicida* ssp. *salmonicida* infections

The enrichment method used in this study was effective for the isolation of phages against *L. garvieae* and *A. salmonicida* ssp. *salmonicida*. With this technique we were able to isolate 22 phages against *L. garvieae* (Table 15) and 34 against *A. salmonicida* ssp. *salmonicida* (Table 16). The *L. garvieae* phages were isolated from water of two fish farms located in the north of Portugal where lactococcosis has been present (in one of the farms we were also able to isolate different strains of *L. garvieae*). The *A. salmonicida* ssp. *salmonicida* phages were isolated from a salt water fish farm in the south of Portugal, from different urban sewage treatment plants and from the Douro river water.

During these isolation trials, we tried to isolate phages against several other bacterial fish pathogens (*Vibrio anguillarum*, *V. ordalli*, *Photobacterium damsela* ssp. *piscicida*, *Tenacibaculum maritimum*, *V. parahaemolyticus*, *Edwardsiella tarda* and *S. parauberis*), however no phages were ever isolated. Only in the case of *Yersinia ruckeri* we were able to isolate a couple of phages from one urban sewage treatment plant. Also, during these isolation experiments we also tried to isolate phages against common human pathogenic bacteria such as *A. hydrophila* and *Escherichia coli*. During these last experiments we were able to isolate more than 100 phages against each of these bacteria, and these isolations were performed in water from fresh and salt water fish farms, Douro river water, urban sewage treatment plants and even mussels to be headed for human consumption.

It was interesting to verify that all phages against *L. garvieae* had to be preserved at -80 °C, otherwise their titer would rapidly decrease, however phages against *A. salmonicida* ssp. *salmonicida* had to be preserved at 4 °C, since preserving them at -80 °C would kill them, even with 50 % glycerol.

Before performing the phage host range, all strains of *A. salmonicida* ssp. *salmonicida* and *L. garvieae* were tested for the presence of temperate phages. However, using the prophage induction technique described none of the bacterial strains showed to be lysogenic. When performing the host range, we verified that no phage was able to infect other bacterial species besides the one they were isolated against (data not shown).

As we can observe from Table 15, from the 21 phages isolated against *L. garvieae*, only three (PPC20.1, PPC31.2 and QS24.1) showed a broad host range against Portuguese strains of this bacterium. However, these phages were not able to infect the Portuguese isolates of farm TC, neither the Spanish nor the type strain.

Table 15 - Host range of the different phages isolated against *Lactococcus garvieae* used in this work: green- complete lysis of the bacterial strain; yellow - incomplete lysis of the bacterial strain; red - resistant bacterial strain.

[illegible]

Table 16 - Host range of the different phages isolated against *Aeromonas salmonicida* ssp. *salmonicida* used in this work; green- complete lysis of the bacterial strain; yellow - incomplete lysis of the bacterial strain; red - resistant bacterial strain.

[illegible]

Table 16 - Continued

[illegible]

The genome size of the three phages was determined by PFGE, however a correct size ladder could not be found, so the exact genome size of the three phages could not be correctly determined (by the lanes in the ladder we are assuming that it should be around 250 Kb) (Figure 29). It was interesting to verify that the three phages showed the same genome size, suggesting that we were dealing with the same phage.

The genome of these three phages was found to be double stranded DNA because it was digested by DNase and restriction endonucleases *Apal* and *Xbal* (Figure 30) but not with RNase A.

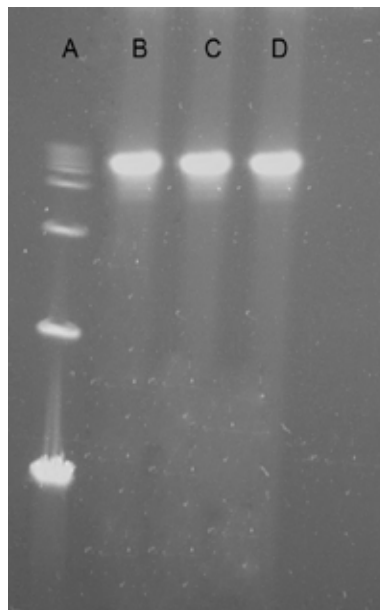


Figure 29 - Pulsed field gel electrophoresis of the genome size of the three phages against *Lactococcus garvieae*. Lane A - MidRange I PFG Marker (New England Biolabs, USA); Lane 2 - Phage PPC20.1; Lane C - Phage PPC 31.2; Lane D - Phage QS 24.1.

As we can observe from Table 16, from the 34 phages isolated against *A. salmonicida ssp. salmonicida*, none showed a broad host range against Portuguese, Spanish or American strains of this bacterium. Because of this lack of broad host no phages were chosen to continue with their characterization.

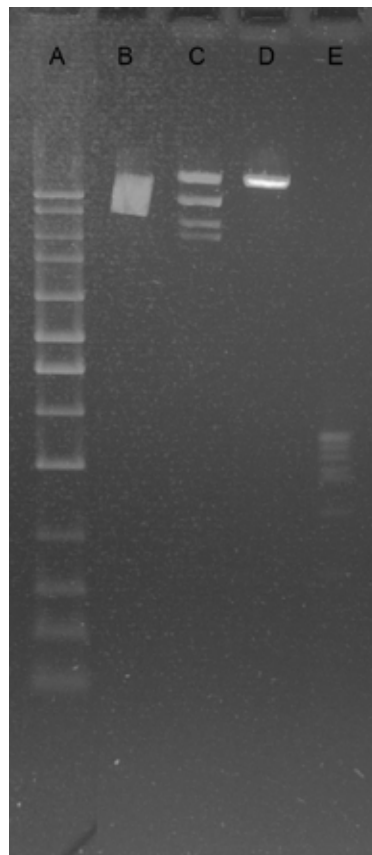


Figure 30 - Restriction pattern of phage PPC20.1. Lane A - Molecular weight marker; Lane B - Restriction fragments of enzyme *SmaI*; Lane C - Restriction fragments of enzyme *ApaI*; Lane D - Restriction fragments of enzyme *XhoI*; Lane E - Restriction fragments of enzyme *XbaI*.

Discussion

Distribution of bacterial fish pathogens in a production facility

In the literature there are not many studies related to bacterial load in pipes, instruments, water or biofilms of a working aquaculture facility, nor a regular evaluation of the efficiency of the filtration systems in use at the installations. From all the data collected in this study the most unexpected value obtained was the high bacterial load observed in the water leaving the nursery tanks (Table 6, P7). This data showed that the nursery water circuit was not working properly, but the reason for this problem was not immediately understood. In fact, only much later was it realised that, on numerous occasions, the night guard would let recirculated water enter the nursery circuit when the low water flow alarm sounded. The abnormal mortalities registered in the larvae disappeared completely only after this action was stopped.

The high bacterial load in the water leaving the nursery tanks, the abnormal mortalities registered in the larvae and the ineffectiveness of ozone skimmer showed that the water circulation in the farm had to be changed and re-evaluated. The first adjustment was in the ozone treatment (the dose was doubled), resulting in a reduction in microbial load in the recirculated water (3 to 4 log reduction in the bacterial counts; data not shown).

Afterwards, the water circuit in the facility was redesigned, existing filters were re-dimensioned, new filters were put in place and the efficiency of the whole system was re-evaluated (Figure 31). These changes allowed a significant reduction of the bacterial load in the water. Thus, the re-dimensioning of the sand and UV filters for the treatment of the hatchery and nursery water allowed the elimination of the countable bacteria (Figure 31, P4). Also, the newly installed sand and UV filters before the adult tanks (Figure 31, P3), allowed the elimination of the countable bacteria. Additionally, the bacterial load in the water to be recirculated through the facility (Figure 31, P6) was greatly reduced by the two newly installed UV filters (Figure 31, P7 and P8).

The analysis of Table 7 shows that *T. maritimum* was isolated from fish tissues in October 2008 and during February, June (four isolates on June 16) and September (five isolates on September 15) of 2009. Isolates were obtained from water in June (one isolate on June 6; three isolates on June 16), July (two isolates on July 7), September (one isolate on September 1; three isolates on September 15) and November 2009 (six isolates from different places in the facility).

The analysis of Table 8 shows that in most cases the presence or absence of *T. maritimum* DNA was the same for both water and sediment/biofilm from the same tank. However, in some cases a negative result in the water was accompanied by a positive result in the sediment/biofilm, suggesting a preferential accumulation of the pathogen in this environment. Despite this, the opposite also occurred (a positive result in the

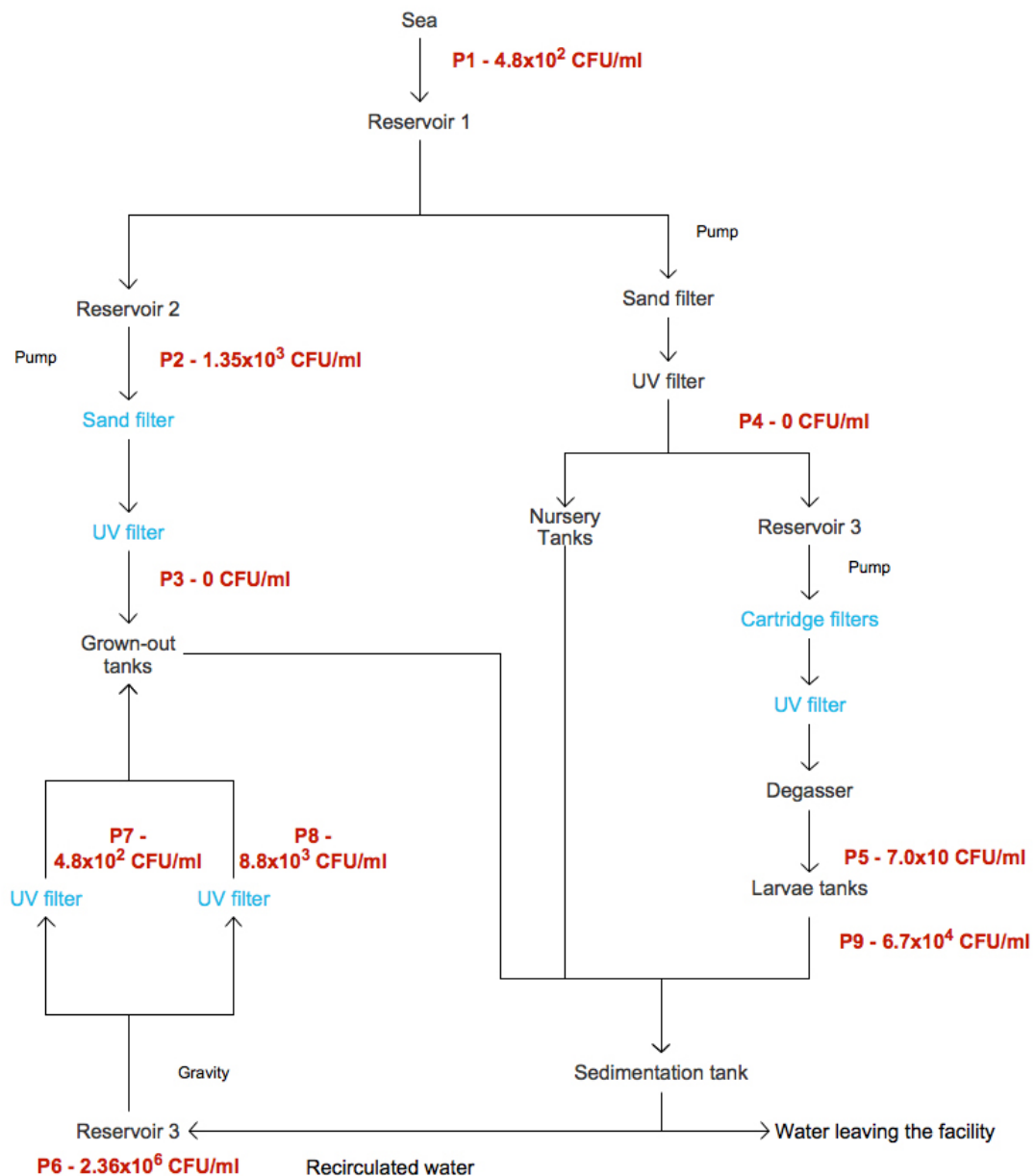


Figure 31 - Schematic representation of the water flow through the facility (arrows), location of the filters (new filters are in blue) and sampling points (P1-P9) after redesign of the water flow. Numbers near the sampling points represent the bacterial load (CFU/ml) in plates of Marine Agar.

water and a negative result in the sediment/biofilm of the same tank), although only twice, in tank T11 in October and in tank T5 in December 2009.

Table 7 also shows that every time *T. maritimum* was isolated from fish, its DNA was also detected in fish mucus by PCR. However, this molecular technique also allowed the detection of the pathogen's DNA in fish mucus on June 6, August 25 and September 8, as well as in November and December 2009, and in January and March 2010, when the isolation from fish tissues was not accomplished, thus suggesting that collection of fish mucus could be used as a non-destructive methodology for analysing and detecting *T. maritimum* in sole by PCR.

Most of the isolations of *T. maritimum* from water were obtained during the summer of 2009; nevertheless in November of 2009, six isolates were obtained from different places in the facility. On July 7, 2009, two isolates were obtained from the water, however the pathogen was neither isolated from fish tissue nor its DNA detected by PCR in fish mucus. This is not really surprising as the pathogen may be present in the water before colonizing the fish surface. Data in Table 7 suggests that fish mucus may be colonized by the pathogen much more easily than its internal organs, and thus once again that the mucus could be a very convenient non-destructive sample for analysing fish for the presence of *T. maritimum*.

Fish from which *T. maritimum* was not isolated, but where its DNA was detected in the mucus, may be survivors of a previous exposure to the bacterium. This is likely, as this bacterium was already known to be present in the facility before the start of this study, and is in agreement with Avendaño-Herrera *et al.* (2006). These authors were only able to recover this bacterium from the mucus of fish surviving exposure to virulent strains of *T. maritimum*. They interpreted this finding as indicative of the possible establishment of a carrier state of the bacterium.

Characterization of Portuguese strains of *Aeromonas salmonicida* ssp. *salmonicida*

Using standard plate and tube tests, all *A. salmonicida* ssp. *salmonicida* strains isolated in Portugal showed complete homology, with the exception of arginine dihydrolase, where 58 % of the isolates gave a negative reaction. This absence of divergence in the phenotypic characteristics found in our isolates is not surprising because *A. salmonicida* ssp. *salmonicida* is known to be a very homogeneous taxon (Toranzo *et al.*, 1991; Holt *et al.*, 1994; Bernoth *et al.*, 1997; Dalsgaard *et al.*, 1994; Austin & Austin, 2012).

The strains of *A. salmonicida* ssp. *salmonicida* used by Sousa (1996) were obtained from one of the farms used in this study (farm CA), however the strains that Sousa used showed a different result for the ONPG reaction. Using a bigger set of strains, Ramos (2006) obtained an ONPG, arginina dihydrolase and lysine decarboxylase different from Sousa (1996), and also found that four of the strains from farm CA did not produce the typical brown pigment of *A. salmonicida* ssp. *salmonicida*. Ramos (2006) suggests that differences between his study and the one from Sousa (1996) could be explained by genetical alterations in the bacterial strains. Ramos (2006) and Sousa *et al.* (1997) also suggest that the isolates causing the epizootic outbreaks could be native Portuguese strains since trout eggs employed in the farm are from their own production.

Using the API 20E system four different numerical profiles (0006104, 1006104, 2006104, 3006104) were obtained with the Portuguese strains of *A. salmonicida* ssp. *salmonicida*. All of these profiles were identified in the API 20E Web V4.1 system as belonging to *A. salmonicida* ssp. *salmonicida*. These same profiles were obtained by Ramos (2006) using a bigger set of Portuguese strains of *A. salmonicida* ssp. *salmonicida*. Also, two of these profiles (2006104 and 3006104) were also obtained by Sousa (1996), as well as an additional third numerical profile (6006104), that we did not obtain even though we studied a bigger set of *A. salmonicida* ssp. *salmonicida* strains.

Using the API 20NE system six different numerical profiles were obtained (5474344, 5474744, 5474754, 5574744, 5574754 and 5574764), all identified in the API 20NE Web V7.0; however two of them (5474754 and 5574754) were identified as *A. hydrophillalcavieae* with the possibility of *A. salmonicida* ssp. *salmonicida*. Very few studies use the API 20NE system for the identification of *A. salmonicida* ssp. *salmonicida* strains. Of all profiles obtained, only one (5574764) was exclusive to the farm SRab (the three strains from this farm originated this same profile). The other five profiles were obtained in the other two farms (IN and CA). This is not unexpected, since during the timeframe of our work animals were transferred from one fish farm to the other. Using a bigger set of Portuguese strains of *A. salmonicida* ssp. *salmonicida*, Ramos (2006) also obtained six different profiles, however he did not obtain profile 5574764, but a different one instead (5430004).

When we compare the results from standard plate and tube test with the ones from the API 20 system, we can observe the existence of false positive and negative reactions. The false negative reactions were obtained with the ONPG test, lysine decarboxylase and arginine dihydrolase (that also originated false positive reactions). Previous studies have showed the existence of false reactions in the identification of *A. salmonicida* ssp. *salmonicida* with the API 20E system (Hahnel & Gould, 1982; Santos *et al.*, 1993; Sousa, 1996; Sousa *et al.*, 1997; El Morabit, 1999; Ramos, 2006). Santos *et al.* (1993) obtained false reactions in the acid production of saccharose, arabinose and sorbitol. Sousa (1996) and Sousa *et al.* (1997), when studying Portuguese strains of *A. salmonicida* ssp. *salmonicida*, obtained false reactions for ONPG, arginine dihydrolase and lysine decarboxylase. El Morabit (1999) using *A. salmonicida* ssp. *salmonicida* isolated from salt water fish obtained false reactions in the acid production of mannose. Studying a bigger set of Portuguese strains of *A. salmonicida* ssp. *salmonicida*, Ramos (2006) also obtained false reactions for ONPG, lysine decarboxylase and arginine dihydrolase. The results in the present study are also in agreement with the ones of Sousa (1996) and Sousa *et al.* (1997), however, contrary to this author, no false reactions were obtained for acid production of saccharose, sorbitol and arabinose, which is in agreement with the results of Ramos (2006).

The *A. salmonicida* ssp. *salmonicida* used in this work were obtained along several months in regular visits to the fish farms. However, no alteration on the antibiotic resistance profile could be observed in relation to time of isolation. The appearance of resistance to nitrofurantoin in isolates of farm IN and CA was expected, since it was extensively used on the farms during the sampling period, at the same time new isolates of *A. salmonicida* ssp. *salmonicida* were being isolated.

It was interesting to verify that strains isolated at the same time, showed different resistance profile, however this has been previously described by Inglis *et al.* (1991) and Sutherland & Inglis (1992). This can become a serious problem in the development of bacterial resistance because if a treatment is started based on the sensibility of one isolate to one antibiotic, the isolates that are resistant can proliferate, and consequently sub-populations resistant to that antibiotic can develop.

Since during the sampling period we observed frequent transfer of animals between farms IN and CA, the different resistance profile to oxolinic acid, oxitetracycline, ampicillin and tetracycline between strains of these farms can look a bit odd. However, this is not that strange if we take into account the results obtained by Inglis *et al.* (1991) and Sutherland & Inglis (1992), that have shown the existence of different resistance profiles in bacteria isolated from the same farm at the same time and sometimes from the same animal. At the same time, we should consider that farm CA is located on a dam and it has been shown that this bacterium can survive for long periods in the environment (water and sediment) (Michel & Dubois-Darnaudpeys, 1980; Austin & Austin, 2012), and the transfer of resistance determinants can occur between bacteria in the sediments (Husevåg & Lunestad, 1995) with subsequent re-infection of fish.

The different resistance profiles against nitrofurantoin, oxytetracycline, ampicillin and tetracycline between the three different Portuguese farms is very interesting, especially if we consider that during the sampling period fish movements occurred among farm IN and CA due to commercial operations but not between IN/CA and SRab. Consequently, it would be more likely that the resistance profile between IN and CA was more similar than between IN/CA and SRab.

Udey & Fryer (1978) showed that virulent strains of *A. salmonicida* ssp. *salmonicida* possess an additional layer beyond the cell membrane. This layer was initially designated “A-layer” and later “S-layer”, and it was shown that it enhanced certain physical attributes of the bacterium by increasing the cellular hydrophobicity and protection, cell-to-cell aggregation, and cell-to-tissue adhesion (Garduño *et al.*, 1995). The S-layer is composed of a single protein subunit (Chu *et al.*, 1991; Sára & Sleytr, 2000), and the gene that codifies it is called *vapA* (virulence array protein gene A) (Belland & Trust, 1987). Based in this gene Gustafson *et al.* (1992) developed a PCR assay for the detection of *A. salmonicida* ssp. *salmonicida*. However, Byers *et al.* (2002) showed that this primer set (AP primers) failed to identify all of the *A. salmonicida*

ssp. salmonicida isolates and suggested that this could be related to the primer target site: the *vapA* gene, which encodes the unique subunit protein (the 'A-protein') of the S-layer of *A. salmonicida ssp. salmonicida* (Chu *et al.*, 1991). This could be the reason why one of the isolates from farm CA (CA5.1.99) and the type strains were not identified as *A. salmonicida ssp. salmonicida* when using the AP primers, but when using the MIY primers the expected PCR amplification product was present. Ramos (2006) showed that even though these two strains were not identified as *A. salmonicida ssp. salmonicida*, when using the AP primers they were able to produce the S-layer, since they originated blue colonies when using Coomassie Brilliant Blue medium and orange colonies with Congo Red Agar (these two media are regularly used for the isolation of *A. salmonicida ssp. salmonicida*, since they are able to connect to the A-protein of the S-layer giving the blue or orange color to the colonies).

Using the Kado & Liu (1981) extraction method, all Portuguese strains of *A. salmonicida ssp. salmonicida* showed the presence of three small plasmids of 3.4, 3.5 and 3.6 Kb. However four different profiles could be established based in the presence of other plasmids; most of the Portuguese strains had the three small plasmids and an additional one of 7.5 Kb (profile I). Profile II (only the three small plasmids) was obtained with two Portuguese strains (CA1.1.99 and CA4.1.99) and a Spanish strain (Isla 2002). All Portuguese strains from farm SRab, the type strain and one Spanish strain (ACR173.1) originated profile III (the three small plasmids and another of 5 Kb). Finally profile IV (the four plasmids of profile I and another of 13 Kb) was obtained with two Portuguese strains (CA6.8.99 and CA6.14.99). Using this method, no open circular (OC) forms were observed contrary to what has been published (Sousa, 1996; Sousa *et al.*, 1997; Toranzo *et al.*, 1991). However, these OC forms could explain to some extent the presence of three sets of three plasmids obtained with the method of Birnboim & Doly (1979). Plasmid content in *A. salmonicida ssp. salmonicida* has been the subject of some debate. Belland & Trust (1989) found that *A. salmonicida ssp. salmonicida* possessed a very similar plasmid content comprised of a single large (70–145 Kb) plasmid and three low molecular weight ones. Livesley *et al.* (1997) reported that five plasmids were most common among the 18 isolates examined; Giles *et al.* (1995) found four or six plasmids with four smaller plasmids of 4.3–8.1 Kb being often observed in isolates from the Atlantic coast of Canada, but six plasmids of 4.2–8.9 Kb among cultures from the Pacific coast of Canada. A total of 23 plasmids and 40 different plasmid profiles were recognized among 124 isolates from Denmark, Norway, Scotland and North America (Nielsen *et al.*, 1993). An earlier theme was repeated insofar as all isolates had one large plasmid of 60–150 Kb, and two low molecular weight plasmids of 5.2 and 5.4 Kb. In addition, two plasmids of 5.6 and 6.4 Kb were frequently present (Nielsen *et al.*, 1993). A larger investigation of 383 isolates over a 6 year period concluded that 1–4 plasmids of 52–105 mDa were inevitably

present, casting doubt on the relevance of plasmid typing for the epizootiology of *A. salmonicida* ssp. *salmonicida* (Sørum *et al.*, 1993b). Oxytetracycline and streptomycin resistant isolates from the Atlantic and Pacific coasts contained four or six plasmids, with four smaller plasmids of 4.3–8.1 Kb being often observed. Some slight variation in plasmid content was noted between sources of the isolates (Giles *et al.*, 1995).

Nielsen *et al.* (1993) regarded plasmid content in general as a method of limited value as an epidemiological marker of *A. salmonicida* ssp. *salmonicida*, but possibly a useful tool for epidemiological studies in certain geographical areas. The usefulness of this method for studying the epidemiology of furunculosis in Portugal could not be established since most of the strains from the different farms showed the same plasmid profile.

RAPD-PCR has been used as an epidemiological tool with some success with other bacterial fish pathogens, such as *Lactococcus garvieae*, *Streptococcus parauberis*, *Pseudomonas anguilliseptica*, *Vibrio tapetis*, *Tenacibaculum maritimum* and *Flavobacterium psychrophilum* (Ravelo *et al.*, 2003; Romalde *et al.*, 1999; Magariños *et al.*, 2000; López-Romalde *et al.*, 2003; Romalde *et al.*, 2002; Chakraborty *et al.*, 1997; Avendaño-Herrera *et al.*, 2004). RAPD-PCR was first applied to the study of *A. salmonicida* ssp. *salmonicida* strains by Miyata *et al.* (1995); these authors showed the presence of identical RAPD profiles in all analysed strains from the United States, England and Japan. Hänninen *et al.* (1995), using three different primers also showed the existence of high genomic homogeneity in strains of this bacterium using the RAPD technique. However, they were able to establish two different clusters, which were related to the origin of isolation of the strains: the first cluster grouped together most of the North American strains, while the second cluster grouped together most of the northern European strains.

In our study, the Spanish, American and most of the Portuguese strains belonged to the same RAPD type, which is in agreement with the results of Miyata *et al.* (1995). The remaining Portuguese strains belonged to different RAPD types, which could not be related to place, time or organ of isolation. So, this technique does not seem to be ideal for epidemiological studies of furunculosis in Portugal, since the RAPD patterns produced were generally homogeneous, and most strains belong to one single RAPD type. Most of Portuguese strains showed the presence of the same four fragments (230, 320, 400 and 750 bp), and although the presence of RAPD-PCR fragments of a particular size has been considered as a species-specific marker, Oakey *et al.* (1998) and Rieseberg (1996) showed that such fragments may not necessarily have the same sequence when amplified from different strains of the same species.

Genomic fingerprinting of bacteria by amplification of repetitive elements has been widely used for the characterization, identification and differentiation of strains in

various fields of microbiology.

Several genera of bacteria have been successfully typed using REP-PCR and ERIC-PCR (Hulton *et al.*, 1991; Rademaker *et al.*, 2000; da Silveira *et al.*, 2002; Bruant *et al.* 2003; Beaz-Hidalgo *et al.*, 2008), with previous studies considering ERIC-PCR best for typing *Aeromonas* spp. (Soler *et al.*, 2003; Szczuka & Kaznowski, 2004; Figueras *et al.*, 2005). ERIC-PCR has been considered to have more discriminatory power than REP-PCR or RFLP when analyzing isolates of *A. popoffi* (Soler *et al.*, 2003). A combination of the REP-PCR and ERIC-PCR methods showed a tendency to cluster strains according to their geographical origin but some isolates from the same place were of different types, indicating that different clones exist within the same geographic location. Another study (Szczuka & Kaznowski 2004) that analyzed *Aeromonas* spp. also found ERIC-PCR to have more discriminatory power than REP-PCR, obtaining an excellent correlation among genera with RAPD analyses. Beaz-Hidalgo *et al.* (2008) found that REP was able to differentiate between three subspecies of *A. salmonicida* analyzed (*A. salmonicida* ssp. *masoucida*, *A. salmonicida* spp. *achromogenes* and *A. salmonicida* ssp. *salmonicida*). However, ERIC was only able to distribute the three sub-species into two genotypes, differentiating *A. salmonicida* ssp. *salmonicida* from the other two subspecies. In agreement with previous studies with aeromonads (Soler *et al.*, 2003; Szczuka & Kaznowski, 2004; Figueras *et al.*, 2005), our results suggest that REP-PCR is more discriminative than ERIC-PCR in the analysis of *A. salmonicida* ssp. *salmonicida*, since one of the ERIC types included all the Portuguese isolates from farm SRab. However, in this study neither REP-PCR nor ERIC-PCR techniques were able to differentiate *A. salmonicida* ssp. *salmonicida* strains based on their geographic origin, time or organ of isolation since both techniques clustered together most of the Portuguese, Spanish and North American strains, which is in agreement with the work of Beaz-Hidalgo *et al.* (2008).

BOX elements have been found in various bacterial species (Stackebrandt, 2006). BOX-PCR was successfully applied to differentiate between strains of *Streptococcus pneumoniae* (Overweg *et al.*, 1999; van Belkum *et al.*, 1996), *Bacillus anthracis* and *B. cereus* (Kim *et al.*, 2002), *Bifidobacterium* species (Masco *et al.*, 2003), members of *Streptomyces* (Lanoot *et al.*, 2004) and *Aeromonas* species (Tação *et al.*, 2005; Skrodenytė-Arbačiauskienė *et al.*, 2010). Tação *et al.* (2005) shows that BOX-PCR fingerprinting is applicable for *Aeromonas* spp. and it could be a useful complementary tool for epidemiological studies of the members of this genus.

Even though most of the Portuguese strains of *A. salmonicida* ssp. *salmonicida* characterized in this study showed the same banding pattern, independently of the place or time of isolation, this technique could be useful for epidemiological studies of this bacterium, since when we consider the Spanish and North American strains we were able to establish three different clusters, based on their geographic origin; the first

group included most of the Portuguese strains, the second most of the North American strains and the third most of the Spanish strains. However, the number of North American and Spanish strains used was relatively low, and strains from other regions of the world were absent, therefore, future work with higher number of strains from different origins is needed to confirm the applicability of these findings. Also, it should be interesting to include in a future work strains belonging to the other subspecies of *A. salmonicida* (*A. salmonicida* ssp. *smithia*, *A. salmonicida* ssp. *pectinolytica*, *A. salmonicida* ssp. *masoucida* and *A. salmonicida* ssp. *achromogenes*).

In this study we have showed that although some genetic variability exists within *A. salmonicida* ssp. *salmonicida*, only BOX typing showed some usefulness as an epidemiological tool for the analysis of the Portuguese strains of this bacterium.

Characterization of Portuguese strains of *Lactococcus garvieae*

Lactococcus garvieae can be retrieved from a variety of sources such as fishes, crustaceans, mammals and derived foods. In fish, lactococcosis has become increasingly widespread after outbreaks occurred all around the world, causing significant economic losses in trout farms specially during the summer months when water temperature rises above 15 °C, and Portugal has not been an exception (Pereira *et al.*, 2004).

In the present study the external and internal clinical signs presented by all fish analyzed are in accordance with those previously described for lactococcosis outbreaks in trout (Domenech *et al.*, 1993; Pereira *et al.*, 2004; Vendrell *et al.*, 2006; Savvidis *et al.*, 2007; Soltani *et al.*, 2008; Sharifiyazdi *et al.*, 2010; Austin & Austin, 2012). It was interesting to verify that, with no clear clinical or pathological signs of lactococcosis and at low water temperatures (10 °C) the bacterium could be isolated from the eye but not from the internal organs of fish. This was a very interesting finding, suggesting that *L. garvieae* can reside in the eyes of fish during the cold months. This result has been previously described by Savvidis *et al.* (2007). Also, Vendrell *et al.* (2006) raises the possibility of the existence of asymptomatic carriers.

Using standard plate and tube tests, all Portuguese isolates of *L. garvieae* had a high level of biochemical homogeneity, regardless of their origin of isolation. These results are comparable to the ones published by other researchers (Sharifiyazdi *et al.*, 2010; Austin & Austin, 2012; Altun *et al.*, 2013). However, the acid producing from lactose, mannitol and maltose were different to what was published (Ravelo *et al.*, 2001; Sharifiyazdi *et al.*, 2010), even though Çağırman (2004) and Soltani *et al.* (2008) got variable results for acid production from lactose. The VP reaction is one of the tests

where more variability exists for *L. garviae* strains all over the world, but all Portuguese strains were negative to VP, which is both in agreement with the results of Sharifiyazdi *et al.* (2010) and Soltani *et al.* (2008) but contrary to those of Eldar *et al.* (1999), Çağırman (2004) and Austin & Austin (2012).

When applying the method developed by Carson *et al.* (2001) to *L. garviae*, an agreement was observed between the conventional and this miniaturized system, with the only exception being the reduction of nitrates. All miniaturized reactions gave clear-cut reactions, so no ambiguity existed. This was the first time this method was applied to *L. garviae*, and it can be useful in the identification of this bacterium, especially when analyzing many isolates, because there is a cost and space saving and one of the best characteristics of the miniaturized format is the speed at which tests become positive (24 to 48 h).

The use of API 20Strep system for the identification of Portuguese isolates of *L. garviae* points to the need to standardize the culture medium, concentration of inoculum, time and temperature of incubation. Even though these systems have been used for the identification of *L. garviae* for a long time, only Colorni *et al.* (2003) refers the time at which results should be read (72 h). Using the previously described standardization we could observe a more precise identification of the isolates, since most of them were identified as *Lactococcus lactis* ssp. *lactis* after 24 h of incubation at 25 °C with a standardized concentration of inoculum and culture medium. Since *L. lactis* ssp. *lactis* and *L. garviae* coincide in the majority of phenotypic characteristics, this kind of result is to be expected. The results obtained in this study are comparable to those of Çağırman (2004), since in both studies the same numerical profile (7143110) was obtained. This same numerical profile was obtained in the isolates from three of the fish farms analysed (NFM, TG and TC). This fact could be explained by the small number of isolates tested in each farm (two in each). In farm PC, a second profile was obtained (7142110) and for farm TPV, independently of the year of isolation, three profiles were obtained; the third profile (5042010) was only obtained with one isolate.

In agreement with what was previously published (Ravelo *et al.*, 2001; Pereira *et al.*, 2004) there is a need to standardize the culture medium, concentration of inoculum and time of incubation when using the RAPID 32 Strep system. When we raised the time of incubation from 4 to 24 h the number of numerical profiles diminished from six (one of them incorrectly identified as *Enterococcus faecalis*) to two (30323101131 and 30333101131), both of which correctly identified as *L. garviae*, with excellent (99.9%) and very good identification (99.4%), respectively. The profiles obtained in this study are different to the ones obtained by Pereira *et al.* (2004) (30333111111, 30337131111), both of which had a low discriminative identification and a non-acceptable profile, respectively. However, in this study the profiles gave more than 99.4 % identity (excellent and a very good identification). Pereira *et al.* (2004) attributed the failure of

the RAPID ID 32 Strep database to identify the isolates as *L. garvieae* to their ability to hydrolyze hippurate, which has been described as uncommon for this microorganism (Collins *et al.*, 1983; Elliott *et al.*, 1991; Colorni *et al.*, 2003), but several authors have reported that some strains are positive for this character (Cheng & Chen, 1998; Ravelo *et al.*, 2001). In our study all Portuguese strains were negative to this test using the RAPID 32Strep system but positive in the API 20Strep system. This is the first time that different results are observed for this character in the API 20Strep and RAPID 32Strep systems. In addition, variability in the utilization of carbohydrates or in the presence of enzymes has been widely described elsewhere (Eldar *et al.*, 1999; Ravelo *et al.*, 2001; Vela *et al.*, 2000). However, in our study only acid production from lactose showed some variability, with 17 % of the isolates positive to this character. This is contrary to what has been described for acid production from lactose using RAPID 32Strep system (Pereira *et al.*, 2004; Ravelo *et al.*, 2001).

In four of the farms (NFM, PC, TG and TC) the same numerical profile (30333101131) was obtained for RAPID 32Strep system. Only in farm TPV, independently of the year of isolation, we obtained the two numerical profiles (30333101131 and 30323101131). Pereira *et al.* (2004,) also obtained different numerical profiles from the different farms analysed: profile 30333111111 was obtained with isolates from farms B and C and profile 30337131111 was obtained with isolates from farms A and D. Even though we can not establish the relation, it is important to note that some of the farms analyzed by Pereira *et al.* (2004) were also analyzed in this work. The differences obtained with the RAPID 32Strep system between the two works can be explained by the fact that, with the exception of farm NFM, all the eggs and/or fingerlings used in the remaining farms originate from imports from other European countries.

Biochemical identification was confirmed by PCR and all the isolates used in this study gave the expected 1100 bp PCR amplification product, which is specific for *L. garvieae* (Zlotkin *et al.*, 1998).

When the phenotype of the bacterial isolates does not provide a suitable level of discrimination, genomic analysis has become the preferred means of investigation for determine bacterial strain relatedness, especially in epidemiological studies. Several techniques have been developed, with Pulsed-Field Gel Electrophoresis (PFGE) being considered the one with highest discriminatory power for strain identification (Olive & Bean, 1999). However, it requires considerable technical expertise, expensive equipment and it takes more time to execute than PCR based typing methods such as RAPD, REP and ERIC-PCR assays. REP and ERIC-PCR are derivatives of RAPD analysis, being based on repetitive elements within the bacterial genome. It was originally used as a tool for strain identification by Versalovic *et al.* (1991). Koeuth *et al.* (1995) identified additional conserved sequences within bacterial genomes, the BOX elements, expanding the range of primers targeting repetitive sequences with potential for PCR based strain identification. RAPD, REP, ERIC and BOX PCR have

now become widely used tools for epidemiological investigations of a range of human, veterinary and plant bacterial pathogens.

RAPD has been described as a good tool for epidemiological studies in *L. garvieae* (Ravelo *et al.*, 2003; Pereira *et al.*, 2004; Foschino *et al.*, 2008; Altun *et al.*, 2013). Using this technique and primer P5 of the Ready-to-Go RAPD analysis beads, Ravelo *et al.* (2003) were able to describe three genetic groups within this bacterial species: Group A, composed by the Spanish, Portuguese, English and Turkish strains; Group B, composed by the French and Italian strains; and Group C, composed by the Japanese strains. On the basis of the results obtained in this study, all the Portuguese isolates were included within the genetic Group A.

With the use of primer M13 for RAPD analysis it was possible to define three big cluster groups, but no relation to place of isolation could be established. Foschino *et al.* (2008) used primer M13 to characterize *L. garvieae* strains isolated from fish and dairy samples collected in northern Italy. These authors were able to establish five different cluster groups at a high similarity and verified that dairy and fish strains revealed a low genetic relatedness as they are often grouped into distinct clusters. However, all the fish strains grouped together in three of the five cluster groups.

The application of REP-PCR for genotyping *L. garvieae* strains was only performed by Schmidtke & Carson (2003) using primers REP1R and REP2 developed by Versalovic *et al.* (1991). Using these primers, they were able to assign the examined *L. garvieae* isolates to one of the five groups established, based on their place of isolation. These same authors used BOX elements to characterize their strains and found out that this technique allowed the separation of capsular from non capsular phenotypes. Contrary to BOX profiles, REP profiles could not distinguish capsular phenotypes (Schmidtke & Carson, 2003). Before using the (GTG)₅ primer we also tried the REP1D e REP2 primer set developed by Versalovic *et al.* (1991) but no bands were obtained with the isolates of *L. garvieae* used in this study. To our knowledge this is the first work where *L. garvieae* strains were typed by REP-PCR using the (GTG)₅ primer.

The results obtained using this primer seem to indicate a potential use in the epidemiological studies of this bacterium, since we were able to some extent establish REP types related to the year and place of isolation. Although the results obtained indicate a promising potential for typing of *L. garvieae*, specially regarding to the strains time and place of isolation, more studies are needed to confirm this hypothesis. These studies should include a larger set of strains isolated from different geographic origins isolated at different times.

As previously referred, BOX elements were found in various bacterial species (Stackebrandt, 2006), and have been successfully applied to the differentiation of

strains of Gram-positive bacteria such as *Streptococcus pneumonia* (Overweg *et al.*, 1999 ; van Belkum *et al.*, 1996), *Bacillus anthracis* and *B. cereus* (Kim *et al.*, 2002). To our knowledge the application of BOX-PCR for genotyping *L. garvieae* strains was only performed once (Schmidtke & Carson, 2003). These authors suggest that different BOX profiles may be attributed to geographical location of strain isolation and possibly capsular phenotypes, however in our work the capsular phenotypes of the *L. garvieae* isolates were not determined. Using BOX profiling we were able to observe the existence of genetic variability inside *L. garvieae*, but were not able to relate them to the farm or time of isolation contrary to the REP-PCR profiles with the primer (GTG)₅. However if we consider a bigger geographic area (country of isolation), three clear groups could be established in agreement to what was described by Schmidtke & Carson (2003). More studies should be performed using a bigger set of *L. garvieae* strains from different places in the world, to confirm or deny this hypothesis.

The confirmation by other authors of the results obtained in this study with REP-PCR using primer (GTG)₅ and with BOX would be an important step for the understanding of *L. garvieae* epidemiology.

PFGE has been used to characterize different bacterial species due to its high discriminative power (Louie *et al.*, 1996; Kelly *et al.*, 1988; Le Bourgeois *et al.*, 1989; Vela *et al.*, 2000). Vela *et al.* (2000) were able to group the fish isolates of *L. garvieae* in three genetically unrelated clones, one comprising Spanish and Portuguese isolates and the other two composed by the Italian and French strains, respectively. When applying the PFGE technique to Japanese strains of *L. garvieae* isolated from the genus *Seriola*, Kawanishi *et al.* (2005) found a high homogeneity in their PFGE profiles, suggesting a clonal structure for *L. garvieae* in this country. The same kind of result was also obtained by Tejadador *et al.* (2001) and Tsai *et al.* (2012). In our study, we were only able to obtain seven different pulsotypes with the Portuguese strains of *L. garvieae*, which could indicate the existence of a clonal structure for the Portuguese isolates of this bacterium. This is especially true if a cluster group with 0.2 similarity is defined, since with it all but four Portuguese strains of *L. garvieae* are not grouped. This group also includes the Spanish strain. Outside this group we have two Portuguese strains from farm TC (that have a very different pulsotype, the type strain and the other two Portuguese strains from farm TPV (isolated in different years). These results seem to suggest that most of the Portuguese strains show a clonal structure with the exception of the strains from farm TC.

Characterization of Portuguese strains of *Streptococcus parauberis*

The clinical signs and gross histopathology observed in infected turbot closely resembled those observed in streptococcosis (Prieta *et al.*, 1993; Doménech *et al.*, 1996b; Austin & Austin, 2012). The meninges of infected turbot showed the presence of small chains of Gram-positive cocci in the dura-mater, but no bacteria were seen in the brain tissue itself, and the erratic behavior and lethargy of the fish was probably related with the meninges infection. Meningitis was also observed in streptococcal infections in different fish species (Eldar *et al.*, 1995; Stoffregen *et al.*, 1996; Ferguson *et al.*, 2000; Chen *et al.*, 2007). Contrary to what is published for streptococcal meningitis in other animals, the typical ischemia-like lesions were not observed in the brain (Chen *et al.*, 2007). The dominant inflammatory cells were macrophages, and no neutrophils were found in the brain, eyes or visceral organs of the analyzed fishes, in opposition to Roberts (1989) and Fange (1992) that reported large numbers of neutrophils in the early stages of inflammation.

Using the standard plate and tube tests, all *S. parauberis* strains isolated in Portugal showed a complete homology, and these results are generally in agreement to what is published for this bacterium (Toranzo *et al.*, 1994 ; Doménech *et al.*, 1996a).

Using the commercial identification system API 20Strep, all Portuguese strains showed homology originating only two numerical profiles (7060000 and 7060010). However, these results were not consistent with those reported by other authors (Toranzo *et al.*, 1995a; Baeck *et al.*, 2006; Nho *et al.*, 2009; Han *et al.*, 2011), that observed different results and sometimes variable results within the same test. The only results that were common in our work with those published by these authors were the positive reactions for VP, LAP and TRE and negative reactions for α GAL, β GUR, LAP, ARA, RAF, AMD and GLY. These differences could be explained by the fact that we only followed one fish farm during a year, isolating *S. parauberis* every month from different fish with different weight. Also, as reported by Nho *et al.* (2009), these differences in the API 20Strep results could be explained by differences in geographical location or physiochemical factors (e.g. temperature and salinity). As Toranzo *et al.* (1994), we observed discrepancies in the results of SOR and LAC, depending if we used conventional or the API 20STREP system (SOR and LAC were positive in conventional tests and negative in the API system). When we compare the results obtained in this study with the ones of Ramos *et al.* (2012), using the same strains, the differences are due to different results in HIP, ESC, PAL and MAN tests. The explanation for these differences can be the fact that in this study we standardized the inoculum, growing the bacteria in blood agar plates for 18 h at 25 °C and the cell density was adjusted spectrophotometrically to OD = 0.8 (A 580). When we compare

the results obtained with the API 20Strep system after 4 and 24 h incubation, we obtained more reliable results after 24 h of incubation.

In the application of API systems for the identification of *S. parauberis* it seems important to standardize the inoculum, especially with the RAPID 32Strep system, because after 24 h of incubation we observed a reduction in the number of profiles and a more accurate identification of the isolates in the API Database, since all the isolates (with more or less percentage of ID) were identified as *S. uberis* 2, and for a time *S. parauberis* was included in this species (Doménech *et al.*, 1996a). To our knowledge this is the first time that the RAPID 32Strep system was applied to the identification of *S. parauberis* infections in fish. The low number of published works related to this fish pathogen could explain this fact. After the first epizootic outbreaks in Spain in 1993 and following studies (Toranzo *et al.*, 1994; Toranzo *et al.*, 1995a; Doménech *et al.*, 1996a), a vaccine was developed (Romalde *et al.*, 1996; Toranzo *et al.*, 1995b) that protected all the growing cycle of turbot. Since then, no major epizootic outbreaks have been observed.

Biochemical identification was confirmed by serological and PCR identification, with all the isolates used in this study giving a positive reaction in the slide agglutination test, and the expected 884 bp amplification product specific for *S. parauberis*.

Amongst the antimicrobial drugs evaluated, the Portuguese strains of *S. parauberis* were resistant to furazolidon, tetracycline and streptomycin. Although the bacteria were sensitive *in vitro* to ampicilin (10), penicilin (10), erythromycin (10), trimethoprim-sulfametazol (25) and to a lesser extent to cefalotin (30) and ampicilin (10), *in vivo* only erythromycin showed some effectiveness in controlling the disease on the fish farm, but it did not entirely eliminate the streptococcal problem. The ineffectiveness of erythromycin in arresting the streptococcal infection can be explained by the fact that this bacterium can survive in the interior of mononuclear cells (Ramos *et al.*, 2012). Toranzo *et al.* (1994) also observed that, *in vitro*, the bacteria were sensitive to ampicillin, chloramphenicol and erythromycin but these drugs were ineffective in arresting the streptococcal septicemia. The reduced appetite of diseased fish could also explain the ineffectiveness of these antimicrobial drugs, since it would lead to decreased medicated food ingestion, causing the death of the fish and the release of millions of streptococci to the environment that would infect other fish. At the same time, these antimicrobial drugs can have difficulties in reaching bacteria present in macrophages, where they will be protected. All these problems will result on an inefficient treatment, and consequently a larger number of fish will become severely affected. These infected fish will become a risk to the fish population, contributing to the appearance of carriers followed by later rounds of infection by the same streptococci (Zimmerman *et al.*, 1975) and the appearance of antibiotic resistance.

Three major mechanisms of tetracycline resistance exist, which are involved in either active efflux of the drug, ribosomal protection or enzymatic drug modification (Giovanetti *et al.*, 2003). Among the various *tet* genes, the *tet(A)*, *tet(B)*, *tet(C)*, *tet(D)*, *tet(E)*, *tet(G)* and *tet(H)* (Jones *et al.*, 2006; Roberts, 2005) are present in Gram-negative bacteria, whereas the *tet(K)*, *tet(L)*, *tet(M)*, *tet(O)*, *tet(S)* and *tet(W)* are mainly found in Gram-positive bacteria (Roberts, 1996; Roberts, 2005).

Streptococci resistant to tetracycline either use the efflux of the drug by proton antiporters (encoded by the *tet(L)* and *tet(K)* genes) or the ribosomal protection (mediated by the *tet(M)*, *tet(O)* and *tet(S)* genes) (Tian *et al.*, 2004; Roberts, 2005). The *tet(S)* gene has mostly been detected in strains of *Listeria* spp., *Enterococcus* spp. and *Lactococcus lactis*. In the genus *Streptococcus* tetracycline resistant genes have been previously found in tetracycline-resistant isolates of *S. pyogenes* (Hammerum *et al.*, 2004) and *S. parauberis* (Park *et al.*, 2009). In this study we could only detect *tet(S)* gene and only in 18.7 % of the isolates, a similar percentage to the one of Park *et al.* (2009). It was interesting to verify that most of the 2005 isolates (3 in 5) possessed this gene, and only three of the 21 strains isolated in 2004 carried the gene, which seems to indicate that the *tet(S)* gene was spreading in the fish farm.

S. parauberis strains isolated from diseased turbot have showed a total homogeneity in phenotypic characterization (Toranzo *et al.*, 1994; Toranzo *et al.*, 1995). Also, the use of some molecular techniques like analysis of cell envelope proteins or plasmid content have not allowed the discrimination of isolates of this bacterium (Toranzo *et al.*, 1995). Due to this homogeneity, Romalde *et al.* (1999) applied the RAPD technique to strains of *S. parauberis*. Using primer P4 of the Ready-To-Go RAPD Analysis Beads (GE Healthcare), these authors found that although each streptococcal strain showed a particular RAPD profile, the genetic groups established after the analysis of similarity could be strongly related to the farm of isolation of the strains. However when we used the RAPD technique with primer M13 in the Portuguese strains of *S. parauberis* no relationship could be established. Contrary to Romalde *et al.* (1999) we only had isolates from one fish farm, so no relationship to the place of isolation could be established, and even including in this analysis the type and the South Korean strains, no relationship could be established. Contrary to Romalde *et al.* (1999) several strains of *S. parauberis* showed the same RAPD profile.

When we applied the REP-PCR technique to the analysis of the Portuguese strains of *S. parauberis* the same kind of results were obtained: several strains showed the same REP profile, but no relationship between strains could be established. Additionally, the South Korean and the type strains showed the same REP profile, different from all the Portuguese profiles. The same was true for the BOX technique: no relationship between strains could be established. However, with the BOX technique, almost all streptococcal strains showed a particular BOX profile, with the only exception

being the two Korean and five Portuguese strains. The application of BOX-PCR to *S. parauberis* showed a high genetic heterogeneity for this bacterial species.

Phages for treatment of *L. garvieae* and *A. salmonicida* ssp. *salmonicida* infections

Bacterial diseases are a major problem affecting fish farming and most of the mass mortalities reported in trout in Portugal are associated with *A. salmonicida* ssp. *salmonicida* and *L. garvieae* (Saraiva et al., 1989; Sousa, 1996; Sousa et al., 1997; Pereira et al., 2004; Ramos, 2006). Until recently the control of bacterial fish pathogens was done with antibiotics. However the emergence of antibiotic resistance among bacterial pathogens, as shown in this work, and the awareness of adverse effects of antibiotics has led to a need for alternatives to antibiotics in aquaculture.

Phage therapy is one of the approaches that is being tried to control bacterial infections in aquaculture, but there have been contradictory results (Nakai et al., 1999; Park et al., 2000; Park & Nakai, 2003; Imbeault et al., 2006; Verner-Jeffreys et al., 2007; Prasad et al., 2011). One feature that makes phages so attractive is their specificity for a bacterial species or strains of that species (the ones that express specific binding sites). This narrow host range is also a significant challenge for phage therapy, because it can render it ineffective. No known phage is lytic for all strains of a single bacterial species. Consequently, phage therapy will only work if phages used for treatment (single or in cocktails) inhibit newly isolated strains of the bacterial species.

Phages are generally isolated from environments that are habitats for the respective host bacteria (Adams, 1959; Nakai & Park, 2002). Since *A. salmonicida* ssp. *salmonicida* and *L. garvieae* used as main host for phage screening were isolated from a farm environment, aquaculture pond water should be the ideal place for isolation of phages against these bacterial species. This was true for phages against *L. garvieae*, but for phages against *A. salmonicida* ssp. *salmonicida*, urban sewage treatment centers and Douro river were better places of isolation. Phages against *A. salmonicida* ssp. *salmonicida* were also isolated in the water of a salt water fish farm in the south of Portugal, where no outbreaks of *A. salmonicida* ssp. *salmonicida* were ever reported.

The most striking observation during this work was that although a huge effort was applied to isolate phages against several other bacterial fish pathogens, sometimes during bacterial outbreaks on the fish farm, no phages were isolated with the exception of phages against *Yersinia ruckeri*, and only from a urban sewage treatment plant.

From all phages isolated against *L. garvieae* only three showed a broad host range infecting almost all of the tested strains of this bacterium, but as showed by the

molecular characteristics of the phages (same DNA size and restriction pattern), the three phages proved to be the same.

The broad host range of this phage makes it useful for use in the control of *L. garvieae* infections in fish, but new phages will have to be isolated and a phage cocktail will have to be developed, since it was not able to infect all the strains tested. This observation was also made by Park *et al.* (1998) and Park *et al.* (1997), who found that their phages did not work against all strains of *L. garvieae*.

The genome of our three phages was found to be double stranded DNA because it was digested by DNase and restriction endonucleases but not with RNase.

Bacteriophages specific to *L. garvieae* were isolated from aquaculture ponds with outbreaks of lactococcosis. This finding suggests that *L. garvieae*-specific phages exist in aquaculture ponds and may contribute to some degree in lessening the severity or persistence of lactococcosis outbreaks. Since *L. garvieae* have been isolated from elements of the aquatic environment such as mud, sediments and water from fish farms (Vendrell *et al.*, 2006), it is understandable that phages may persist in aquaculture ponds, which is in accordance with the idea that bacteriophages are ubiquitous in the environments inhabited by their respective host(s) (Adams, 1959; Nakai & Park, 2002).

Conclusions

The evaluation of the bacterial load in the water throughout the installations of ACC was essential for detecting the inefficacy of many of the filters, some of which were completely useless, and which led to major changes in the water circuit of the facility.

At the same time, the abnormal mortality rate observed among the larvae would not have been detected if a bacteriological study had not been undertaken. Larvae mortality diminished and gas bubbles stopped being observed when the skimmer placed just before reservoir 2 was taken off the circuit (July 2009). Larvae mortality completely disappeared when the use of recirculated water in the nursery circuit was detected and stopped. These facts point to the usefulness of periodical assessment of the bacteriological quality of water used in fish farming, at representative places in the facility, so that corrective measures can be applied.

Tenacibaculum maritimum was the only fish pathogen isolated in the farm and the only one detected by PCR. This pathogen was widespread throughout the farm since it could be recovered or its DNA detected in samples from fish organs or mucus, water, sediment or biofilm from different tanks in the facility. No other bacterial fish pathogen, or its DNA, could be detected on fish or environmental samples from the fish farm.

Traditional biochemical characterization of *Aeromonas salmonicida* ssp. *salmonicida* showed the complete homology of the Portuguese isolates of this species, with the exception of arginine dihydrolase, where 58% gave a negative result. Using the API 20E system all strains were identified as *A. salmonicida* ssp. *salmonicida*, however when using the API 20NE system not all strains were identified as belonging to this species. Consequently the API 20E system should be used when identifying this bacterial species. The primers developed by Gustafson *et al.* (1992) for the molecular identification of *A. salmonicida* ssp. *salmonicida* should not be used for the identification of this bacterium since they originate false negatives, contrary to those developed by Miyata *et al.* (1996). The molecular characterization of the strains of *A. salmonicida* ssp. *salmonicida* showed the existence of genetic heterogeneity, however only BOX typing was able to cluster most of the strains accordingly to their geographic origin. The other techniques used did not seem to be useful for epidemiological studies of this bacterial species since it was not possible to relate the clusters established with the geographic origin, time of isolation or organs of isolation the strains.

The *Lactococcus garvieae* strains isolated in Portugal showed a high level of biochemical homogeneity, regardless of their origin of isolation. However some biochemical characteristics (acid producing from lactose, mannitol and maltose)

are different from what is published. RAPID 32Strep system seems to be the best miniaturized system for the identification of *L. garvieae*, but care should be taken to standardize culture medium, inoculum density and time and incubation temperature. The molecular characterization of *L. garvieae* showed the existence of genetic heterogeneity in the Portuguese strains of this bacterium. From all the methods applied, REP technique using primer (GTG)₅, allowed to cluster the strains according to farm and year of isolation, and consequently it could be very useful in the epidemiological study of this bacterium. Also, BOX could have some utility in epidemiological studies of this bacterium since clusters, based on their geographic origin, could be established.

The Portuguese strains of *Streptococcus parauberis* showed a high biochemical homology, independently of the system used (traditional, API 20Strep or RAPID 32Strep, even though in the RAPID 32Strep system we observed more variability of results). This could be explained by the fact that all strains came from the same farm. All Portuguese strains were resistant to tetracycline and we found that the *tet(S)* gene appeared to be spreading through the farm.

The *S. parauberis* strains studied showed molecular heterogeneity but none of the techniques applied had epidemiological importance since the types established could not be related to geographic origin, time or organ of isolation.

Three *L. garvieae* phages showed a broad host range, but after genomic analysis it was found that they were all the same, since their DNA had the same size, was double stranded and was identically digested by the restriction enzymes *Apal* and *Xbal*.

From all phages isolated against *A. salmonicida* ssp. *salmonicida* isolates, none showed a broad host range (most of the phages only affected a small number of bacterial isolates). This made impossible to established a typing scheme, and to choose one or more to continue with their characterization.

During this work we encountered significant problems in the isolation of phages against bacterial fish pathogens. Even though we tried to isolate phages against nine different bacterial fish pathogens, we were only able to isolate against *L. garvieae* and *A. salmonicida* ssp. *salmonicida*. The same did not happen with common human pathogenic bacteria such as *A. hydrophila* and *Escherichia coli*. This difficulty in isolating phage against bacterial fish pathogens may hinder the application of phage therapy in aquaculture.

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