UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA VETERINÁRIA

MESOPHILIC *AEROMONAS* IN THREATENED IBERIAN LEUCISCIDS: CONSERVATION AND PUBLIC HEALTH IMPLICATIONS

MIGUEL LUCA AUGUSTO GRILO

Orientador(es): Professora Doutora Maria Manuela Castilho Monteiro de Oliveira

Professora Doutora Joana Isabel Espírito Santo Robalo

Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Veterinárias na especialidade de Sanidade Animal

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To my parents and to Pedro

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Aeromonas mesofílicas em leuciscídeos ibéricos ameaçados: implicações para a Conservação e Saúde Pública

Resumo

Apesar do elevado risco de extinção dos leuciscídeos ibéricos, desconhece-se o impacto das doenças bacterianas na sua conservação. Um conhecimento abrangente sobre a interação entre estas espécies e agentes patogénicos, como *Aeromonas* mesofílicas, bem como o desenvolvimento de medidas de biossegurança para programas *ex situ,* são considerados essenciais para o sucesso da sua conservação. Os principais objetivos desta tese são a avaliação da epidemiologia de *Aeromonas* mesofílicas em *Iberocondrostoma lusitanicum*, bem como a caracterização dos seus perfis de resistência antimicrobiana e virulência, a deteção de diferenças na diversidade de *Aeromonas* spp. de *I. lusitanicum* e *Squalius pyrenaicus*, a avaliação do potencial de medidas de biossegurança no controlo de *Aeromonas* spp. em programas *ex situ* e a testagem do efeito de alterações climáticas em *Aeromonas* spp.

Os resultados mostraram que *I. lusitanicum* apresentaram estado geral de saúde inferior na época seca, variando com a localização. As comunidades de *Aeromonas* variaram entre épocas e localizações, enquanto o seu potencial patogénico aumentou na época seca. Foram detetados fenótipos de resistência relevantes (carbapenemos e fluroquinolonas). Além disso, *I. lusitanicum* e *S. pyrenaicus* apresentaram diferentes níveis de lesões cutâneas, sendo *S. pyrenaicus* a espécie mais afetada. As comunidades de *Aeromonas* diferiram entre as duas espécies de peixes. Adicionalmente, o uso de medidas de biossegurança sugere uma diminuição na prevalência de *Aeromonas* spp. em programas *ex situ*, bem como da sua patogenicidade para peixes, enquanto foi observado um aumento da resistência antimicrobiana ao longo do programa. Finalmente, as alterações climatéricas previstas pelo Painel Internacional sobre Mudanças Climáticas para a temperatura e pH da água influenciaram o crescimento, a produção de biofilme e os perfis de resistência antimicrobiana de *Aeromonas* spp.

Os resultados atuais esclarecem sobre a epidemiologia de *Aeromonas* mesofílicas em leuciscídeos ibéricos ameaçados e contribuem para o estabelecimento de medidas de conservação adequadas.

Palavras-chave: *Aeromonas* spp., *Iberochondrostoma lusitanicum*, *Squalius pyrenaicus*, resistência antimicrobiana, virulência.

Mesophilic *Aeromonas* in threatened Iberian leuciscids: conservation and public health implications

Abstract

Despite the high risk of extinction of the Iberian leuciscids, the impact of bacterial diseases on their conservation is unknown. A comprehensive knowledge of the interaction between these species and pathogens, such as mesophilic *Aeromonas*, as well as the development of biosafety measures for *ex situ* programs, are considered essential for the success of their conservation. The main objectives of this thesis are the evaluation of the epidemiology of mesophilic *Aeromonas* in *Iberochondrostoma lusitanicum*, as well as the characterization of their antimicrobial resistance and virulence profiles, the detection of differences in the diversity of *Aeromonas* spp. of *I. lusitanicum* and *Squalius pyrenaicus*, the evaluation of the potential of biosecurity measures in the control of *Aeromonas* spp. in *ex situ* programs and testing the effect of climate change on *Aeromonas* spp.

The results showed that *I. lusitanicum* presented lower general health status in the dry season, varying with location. *Aeromonas* communities varied between seasons and locations, while their pathogenic potential increased in the dry season. Relevant resistance phenotypes (carbapenems and fluoroquinolones) were detected. Furthermore, *I. lusitanicum* and *S. pyrenaicus* showed different levels of skin lesions, with *S. pyrenaicus* being the most affected species. *Aeromonas* communities differed between the two fish species. Additionally, the use of biosecurity measures suggests a decrease in the prevalence of *Aeromonas* spp. in *ex situ* programs, as well as its pathogenicity to fish, while an increase in antimicrobial resistance was observed throughout the program. Finally, climate change predicted by the International Panel on Climate Change for water temperature and pH influenced the growth, biofilm production and antimicrobial resistance profiles of *Aeromonas* spp.

The current results clarify the epidemiology of mesophilic *Aeromonas* in threatened Iberian leuciscids and contribute to the establishment of adequate conservation measures.

Keywords: *Aeromonas* spp., *Iberochondrostoma lusitanicum*, *Squalius pyrenaicus*, antimicrobial resistance, virulence.

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List of abbreviations

%	Percentage
°	Degree
µg	Microgram
µL	Microlitre
µm	Micrometre
µM	Micromolar
AHLS	Acylated homoserine lactones
AK	Amikacin
AMR	Antimicrobial Resistance
ARB	Antibiotic resistant bacteria
ARGs	Antibiotic resistance genes
ATCC	American Type Culture Collection
ATM	Aztreonam
AUG	Amoxicilin/clavulanic acid
BCS	Body Condition Score
BHI	Brain Heart Infusion
CAZ	Ceftazidime
CCEDCV	Centro de Conservación de Especies Dulceacuícolas de la Comunitat Valenciana
CFU	Colony-Forming Units
CFU/mL	Colony-Forming Units per millilitre
CLSI	Clinical e Laboratory Standards Institute
COS	Columbia Blood
D	Simpson index

DNA	Deoxyribonucleic acid
E	Erythromycin
e.g.	*Exempli gratia*
ENF	Enrofloxacin
ESU’s	Evolutionary Significant Units
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FFC	Florfenicol
g/L	Gram per litre
GLM	Generalized Linear Model
GLMM	Generalized Linear Mixed-Effects Model
GSP	Glutamate Starch Red Phenol
gyrB	DNA gyrase subunit B
h	Hours
HGT	Horizontal gene transfer
i.e.	*Id est*
IMI	Imipenem
IPCC	Intergovernmental Panel on Climate Change
IS elements	Insertion sequence elements
IU	International Unit
IUCN	International Union for Conservation of Nature
L	Litre
LPS	Lipopolysaccharides
m	Metre
MAR	Multiple Antibiotic Resistance
mg/L	Milligram per litre
min	Minute

mL	Millilitre
mm	Millimetre
mS	MiliSiemens
NaCl	Sodium chloride
NI	Nitrofurantoin
nm	Nanometre
NWT	Non-wild-type
ºC	Degree Celsius
OD	Optical Density
OTU's	Operational Conservation Units
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PhD	Philosophy Doctor
ppm	Parts-per-million
PVC	Polyvinyl chloride
RAPD	Random Amplified Polymorphic DNA
RCP	Representative Concentration Pathway
rpm	Revolutions per minute
rpoB	RNA polymerase beta subunit
rRNA	Ribosomal ribonucleic acid
S layer	Surface layer
s	Second
S	Streptomycin
SD	Standard Deviation
SEM	Standard error of the mean
SIBIC	Sociedade Ibérica de Ictiologia

SLS	Skin Lesion Score
sp.	Specie
Spp.	Species
T	Tetracycline
T2SS	Type II secretion system
T3SS	Type III secretion system
T4SS	Type IV secretion system
T6SS	Type VI secretion system
TBE	Tris-Borate-EDTA
T^{o}C	Temperature in degrees Celsius
TS	Sulfamethoxazole/trimethropim
TSB	Tryptic Soy Broth
UK	United Kingdom
UPGMA	Unweighted pair group method with arithmetic average
USA	United States of America
UV	Ultraviolet
V	Volt
vs.	Versus
w/v	Weight per volume
WT	Wild-type
β-lactams	Beta-lactams
μ	Mean

Chapter 1. Introduction

1.1.1. Iberian leuciscids: origins and paths

The family Leuciscidae, belonging to the order Cypriniformes, is considered one of the largest within the suborder Cyprinoidei and encompasses the greatest diversity of species (Eschmeyer et al. 2017; Schönhuth et al. 2018). Their expression is particularly important in freshwater communities in Eurasia and North America, presenting a considerable wide distribution in a variety of habitats (Schönhuth et al. 2018), as well broad features of trophic adaptations and morphology (Kottelat and Freyhof 2007). Generally, species belonging to this family display small body sizes, usually under 25 cm long (Froese and Pauly 2016). In the Iberian Peninsula, the subfamily Leuciscinae is represented by the following genera: *Achondrostoma,* Robalo et al. 2007a; *Anaecypris*, Collares-Pereira 1983; *Iberochondrostoma*, Robalo et al. 2007a; *Parachondrostoma*, Robalo et al. 2007a; *Pseudochondrostoma*, Robalo et al. 2007a; and *Squalius*, Bonaparte 1837 (Robalo et al. 2007a; Sousa-Santos et al. 2019).

Leuciscids are primary fish (i.e., intolerant to salt water) and their habitat distribution is restricted to available freshwater streams (Sousa-Santos et al. 2019). These species present low dispersion success since their distribution and evolutionary history is highly dependent on the biogeographical alterations of the habitats they occupy, i.e., when stream connection is lost, there is no possibility for these species to disperse to new habitats (Robalo et al. 2007a).

European colonisation by leuciscids is believed to have occurred in the Oligocene and originated from Asia, following the disappearance of the Turgai Sea which separated both continents (Robalo et al. 2007a). Further dispersal to the southern peninsulas, such as the Iberian Peninsula, is hypothesized by two dispersion routes. One of the possible routes ("northern-dispersal hypothesis") included gradual colonisations in Mediterranean freshwater bodies through river captures from central Europe before the alpine orogeny changes that originated mountain chains separating southern territories (Banarescu 1992), while the other ("southern sea-dispersal hypothesis") hypothesized colonisations around the Mediterranean Sea during the Messinian salinity crisis and the Lago Mare period (Bianco 1990).

Further evolutionary patterns presented by Iberian Leuciscinae were directly connected with the changes in the freshwater network occurring in the region after the isolation from the rest of Europe following the elevation of the Pyrenees during the Miocene. Speciation and diversification of leuciscids were shaped by connections available between basins that are currently independent and by the transition of a hydrographic network dominated by large

endorheic lakes to exorheism in the late Neogene-Quaternary, with basins starting to drain to the Atlantic Ocean and to the Mediterranean Sea. While evolution occurred slowly during the endorheic phase, the exorheic shift originated a dispersion to the western areas that congregated a high level of cladogenetic events (Sousa-Santos et al. 2019).

These events likely resulted in the freshwater fish scenario that is currently observed in Europe. Contrary to the panorama regarding leuciscid species in central Europe, where a low number of species is present and distributed by a wide set of genera presenting wide distribution areas, in areas such as the Iberian Peninsula, a high level of leuciscid endemism is observed, although few genera are documented and the distribution areas are generally reduced for each species (Robalo 2008).

1.1.1.1. Threats to Iberian leuciscids

Although efforts have been deployed to establish international legislation that aim to guarantee European freshwater networks' quality and sustainability, a high proportion of these habitats are impacted by anthropogenic activities (Costa et al. 2021). Both the importance and impact each threat imposes vary spatially in the European territory, thus compromising the conservation status of European freshwater fishes differentially (Clavero et al. 2010; Hermoso and Clavero 2011). Generally, about 40% of European freshwater fish species are facing risk of extinction. However, regions like the Iberian Peninsula are particularly at risk, with a high number of potential threats reported (Costa et al. 2021). This area is known for harbouring a high level (73%) of fish species' endemicity, typically characterized by restricted distribution ranges (Reynolds et al. 2005; Maceda-Veiga 2013). Furthermore, around 81% (21 out of 26 species) of endemic Iberian leuciscids are classified under a threatened status (IUCN 2021; Table 1).

Several threats have been identified as impacting fish species survival in the Iberian Peninsula. However, the final impact might originate by the synergistic negative effect of the threats or by the action of a specific one, depending on the situation (Maceda-Veiga 2013). According to the IUCN, the main threats to the Iberian ichthyofauna include water extraction and use (e.g., hydrological infrastructures as dams), introduced exotic and invasive species, climate change effects (e.g., extreme droughts and floods), water pollution (e.g., agricultural and forestry effluents) and human exploitation (e.g., aquatic resources harvesting, urbanization) (Prenda et al. 2006; Maceda-Veiga 2013; Sousa-Santos et al. 2016; Costa et al. 2021). Threat levels within the Portuguese and Spanish Red Lists tend to be higher than those reported by the IUCN, reinforcing the importance of updating these national instruments (Miqueleiz et al. 2021).

Table 1. Endemic Iberian leuciscid species and corresponding conservation status. Doadrio et al. 2011; SIBIC 2017; Collares-Pereira et al. 2021; IUCN 2021. * Hybridogenetic complex, ** Iberian and French endemism.

Genus	Species	IUCN category
Achondrostoma	*arcasii* (Steindachner 1866)	Vulnerable
	occidentale (Robalo et al. 2005)	Endangered
	oligolepis (Robalo et al. 2005)	Least Concern
	salmantinum (Doadrio and Elvira 2007)	Endangered
Anaecypris	*hispanica* (Steindachner 1866)	Endangered
Iberochondrostoma	*almacai* (Coelho et al. 2005)	Critically Endangered
	lemmingii (Steindachner 1866)	Vulnerable
	lusitanicum (Collares-Pereira 1980)	Critically Endangered
	olisiponensis (Gante et al. 2007)	Critically Endangered
	oretanum (Doadrio and Carmona 2003)	Critically Endangered
Parachondrostoma	*arrigonis* (Steindachner 1866)	Critically Endangered
	miegii (Steindachner 1866)	Least Concern
	turiense (Elvira 1987)	Endangered
Pseudochondrostoma	*duriense* (Coelho 1985)	Vulnerable
	polylepis (Steindachner 1864)	Least Concern
	willkommi (Steindachner 1866)	Vulnerable
Squalius	*alburnoides* (Steindachner 1866) *	Vulnerable
	aradensis (Coelho et al. 1998)	Vulnerable
	carolitertii (Doadrio 1987)	Least Concern
	castellanus (Doadrio et al. 2007)	Endangered
	laietanus (Doadrio et al. 2007) **	Least Concern
	malacitanus (Doadrio and Carmona 2006)	Endangered
	palaciosi (Doadrio 1980)	Critically Endangered
	pyrenaicus (Günther 1868)	Endangered
	torgalensis (Coelho et al. 1998)	Endangered
	valentinus (Doadrio and Carmona 2006)	Vulnerable

Although water extraction is frequent in freshwater ecosystems worldwide, it is especially problematic in arid and semi-arid regions such as the Iberian Peninsula (Clavero et al. 2010). The ratio of water use and availability in the peninsula increases in a southern direction, being generally high in this area (contrary to the northern region which is considered a wet region) (Maceda-Veiga 2013). This problem is even exacerbated by illegal extractions (Hermoso and Clavero 2011). Additionally, several hydrological infrastructures like weirs and dams were built in the past century to aid agricultural irrigation, hydropower production, domestic consumption and flood regulation (Prenda et al. 2006). However, such structures alter freshwater network's function (e.g., changes in water flow and temperature oscillations) and impact biodiversity life-traits (e.g., movement restriction and spawning areas destruction) (Maceda-Veiga 2013; Duarte et al. 2021).

In the Iberian Peninsula, non-native species compose almost half of the freshwater fish species in region (Clavero et al. 2010; Maceda-Veiga 2013; Costa et al. 2021). This is considered one of the main drivers of native species extinction and declines (Leunda 2010; Hermoso et al. 2011), although it is difficult to disentangle the cumulative effect habitat alterations also have. Climatic alterations are expected to accentuate the pressures of exotic species on native communities (Costa et al. 2021). The introduction of exotic and invasive species results in direct consequences such as predation, competition for resources, hybridization, agonistic behavioural interactions and the possible transfer of infectious agents (Leunda 2010; Almodóvar et al. 2012; Almeida et al. 2014; Sousa-Santos et al. 2018). Indirectly, fish introductions can also result in poor water quality due to changes in nutrient cycling (Maceda-Veiga 2013).

Water abstraction is also extremely important in the Iberian Peninsula, like in the rest of the regions having a Mediterranean-type climate, where the proportion of fish species affected by water scarcity is the highest in all the areas accessed by the IUCN (Clavero et al. 2010). Droughts, particularly incident in warmer months, and the consequent reduced river flow affect native fish community's abundance, diversity, size structure, growth, spawning and recruitment (Merciai et al. 2017; Costa et al. 2021). Although fish species native to these regions are accustomed to these water level and temperature oscillations, the expected amplified effect of climate changes in the frequency and severity of the droughts will likely severely affect these species and is expected to occur with greater impact in Southern Europe (Jaric et al. 2019). This is demonstrated by behavioural alteration in native fish fauna due to heatwaves (Mameri et al. 2020a). Additionally, and since fire frequency is expected to increase in the Mediterranean region due to climate change (Mouillot et al. 2002), fires are also an important threat in the region, decreasing the stability of the river channel, increasing the concentration of toxic compounds and altering water temperature (Maceda-Veiga 2013),

resulting in fish abundance and behavioural alterations in burnt areas (Sostoa et al. 2003; Gonino et al. 2019).

Pollution of the freshwater streams is an additional significant threat, with the majority of the Iberian freshwater networks presenting historical records of high pollution levels due to anthropogenic activities. Although the modernization of sewage systems substantially improved water quality, current sewage treatment plants still fail to remove several pollutants and to prevent their introduction in aquatic habitats. Additionally, atmospheric deposition and terrestrial run-offs are important pollutant introduction routes in freshwater ecosystems (Maceda-Veiga 2013). In fact, agricultural and industrial effluents affect half of Europe's freshwater ecosystems (Costa et al. 2021).

Furthermore, habitat degradation and destruction is enhanced by multiple anthropogenic activities. Apart from those already described, mining for gravel extraction severely impacts the hydrological and ecological functions of river beds, directly impacting fish species by reducing possible breeding habitats for native species (Maceda-Veiga 2013). Additionally, habitat management policies often consider solutions that best fit the urbanization or agricultural models, without taking into consideration native species requirements (Maceda-Veiga 2013). For instance, riparian coverage is essential for river functioning (i.e., stabilization of river margins, pollutant filter, nutrient source) and for native species (e.g., water temperature control, UV radiation filter) (Elosegui et al. 2011). The indiscriminate removal of riparian shading or the substitution of autochthonous species with exotic ones (i.e., for agricultural purposes or by invasion) fail to provide the functions previously described and have detrimental effects in water quality and fish survival (Maceda-Veiga 2013).

Consequences for fish health can be met from the above-mentioned threats. While water abstraction is associated to decreasing health conditions and susceptibility to disease in native species (Maceda-Veiga et al. 2009; Sánchez-Hernández 2017; Maceda-Veiga et al. 2019), environmental degradation and water pollution is also associated with similar outcomes (Austin 1998; Noga 2000; Law 2001; Benejam et al. 2010). Additionally, the role of exotic species as vectors of pathogenic agents and the examples of fish pathogens introduced in Iberian freshwaters is concerning (Maceda-Veiga et al. 2009; Gozlan et al. 2010).

1.1.1.2. Conservation measures

Since Iberian leuciscids, as well as other Iberian freshwater fish species, present such a poor conservation status globally, the continuous development and implementation of conservation measures that counteract their extinction risk is aimed (Maceda-Veiga 2013). Since rivers are by definition open systems with continuous unidirectional flow, in which a

threat occurring in one place may impact communities established in a different point, and the fact that the impact from the described threats is shared by the majority of the fish species, efforts in improving ecological processes should be favoured over single species conservation, although case-to-case reflection needs to be addressed (Maceda-Veiga 2013; Costa et al. 2021). Hence, the development of large reserve networks to protect biodiversity, such as the Natura 2000, has been stressed at an international level. However, freshwater biodiversity coverage of this network is low (less than 20%) and fails to achieve a conservation target of 25% for more than 80% of the species contemplated in the legislation (Hermoso et al. 2015). Moreover, basin type hinders different prospections for fish biodiversity, since larger basins in the Iberian Peninsula congregate a higher number of native, endemic and rare species, but smaller basins present higher community conservation values due to lower degree of invasion by non-native species (Clavero et al. 2004).

Following recommendations by the IUCN and expert working groups, the conservation management of Iberian leuciscids should include the implementation of measures to improve habitat quality, minimize the impact of non-native species and guarantee fish stocks sustainability by *in situ* and *ex situ* programs (Maceda-Veiga 2013).

Habitat management and restoration (Figure 1) aggregates several methodologies that should be contemplated as a holistic strategy to improve habitat quality for fish species. Governmental efforts have been deployed to restore rivers, including the removal of dams (Rodeles et al. 2017). However, dam removal can be met with high social and economic costs, and its planification should be performed in parallel to the study of river and populations connectivity with appropriate indexes as a method for dam removal selection and ecological corridors creation for endangered species (Rodeles et al. 2021). Also, dam removal not always guarantees native fish communities restoration (Gregory et al. 2002; Johnson et al. 2008). So, focus in removing obsolete physical barriers and creating fish passages in small tributaries can often be particularly successful since native fish populations may persist in these ecosystems (Maceda-Veiga 2013). Regarding large dams, planned flow and sediment discharges in accordance with the native species life-history can help to guarantee river function (Rovira and Ibañez 2007). Fish communities would also benefit from gravel bedforms and artificial riffles creation for ensuring river depth and flow variability (Santos et al. 2018). These measures should be accompanied by water management policies and supervision that guarantee the reduction of legal and illegal water extraction and a system of water re-use (Maceda-Veiga 2013).

Riparian coverage management also assumes an important role in the habitat restoration. Although complete removal of invasive species or plantations could be impracticable and costly, as well as creating abrupt changes in aquatic communities (Elosegui

et al. 2011), the creation of riparian buffers composed of native vegetation mixed with plantations can mitigate litter input created by plantations alone, reinforce the stability of the river margins and provide cover important for fish communities (Maceda-Veiga 2013; Santos et al. 2018).

Finally, water quality improvements can be met with raising river pollution awareness, either by encouraging more ecological agricultural practices with economic incentives or increasing sewage treatment plants capacity to reduce illegal disposal of domestic and industrial effluents. As a safety measure, the creation of adjacent lagoons can guarantee fish escape and survival during acute pollution events in signalled high-risk areas (Maceda-Veiga 2013).

Figure 1. Habitat restoration work developed in Sizandro river under the framework of the *Achondrostoma occidentale* conservation program. Left – November 2018, right – June 2020. Photo by Carla Sousa-Santos.

The implementation of programs to control and eradicate exotic and invasive fish species (Figure 2) is an additional important conservation measure. Although complete eradication is usually impracticable and costly and might affect trophic interactions already established in Iberian freshwaters with other taxonomic groups (Tablado et al. 2010), local control plans using electrofishing, nets and traps applied in smaller areas of high conservation value can present a better cost-success ratio (Maceda-Veiga 2013). However, prevention of introductions is fundamental and relies in two pillars: awareness and modelling of invasion potential. The combined efforts of educational programs with target populations (e.g., anglers) and regulation that forbids the exploitation of possible invasive species in Iberian waters, imposes specialized training prior to permits to fish/handle these species and enforces legislation regarding the introduction of exotic species (e.g., banning live-bait use) should be

pursued (Olden et al. 2011; Maceda-Veiga 2013). Furthermore, the use of models that help to predict invasion success of exotic species before introduction in Iberian rivers should be paramount to the previously described measures (García-Berthou 2007).

Figure 2. *Procambarus clarkii* individuals collected during programs for the species controls in Portuguese streams. Photo by Carla Sousa-Santos.

Ex situ breeding programs (Figure 3) targeting Iberian leuciscids have been essential for the *in situ* conservation of native populations (Gil, Sousa-Santos and Almada 2010; Sousa-Santos et al. 2014b). This is considered a solution for populations whose sustainability is in jeopardy due to low stocks and severe habitat degradation (Gil, Sousa-Santos and Almada 2010). Currently, several programs have been conducted in Portugal and Spain to guarantee the increase of captive stocks of Iberian leuciscids and posterior release into the wild. These efforts to reinforce wild endangered populations have included species such as *Achondrostoma arcasii*, *Achondrostoma occidentale*, *Achondrostoma salmantinum*, *Anaecypris hispanica*, *Iberochondrostoma almacai*, *Iberochondrostoma lemmingii*, *Iberochondrostoma lusitanicum*, *Parachondrostoma arrigonis*, *Pseudochondrostoma duriense*, *Pseudochondrostoma polylepis*, *Pseudochondrostoma willkommii*, *Squalius alburnoides* (hybridogenetic complex), *Squalius aradensis*, *Squalius pyrenaicus* and *Squalius torgalensis* (Sousa-Santos et al. 2014b; CCEDCV 2019; Marcos, Santos and Santos 2019; Diario de Madrid 2021; Junta de Extremadura 2021). It is important to mention that captive breeding should be implemented in parallel to habitat restoration to guarantee preservation of restocked populations and the mitigation of the threats that were responsible for their extinction risk and that justified their inclusion as targets for the *ex situ* conservation program

(Sousa-Santos et al. 2014b). Also, captive breeding programs should guarantee that individuals raised in captivity maintain the natural behaviours of the species that will be essential after their release into the wild, that captive conditions present bio-safety measures that prevent disease outbreaks and further dissemination events into the wild and that the breeding conditions mitigate eventual changes to the original genetic pool of the target populations (Sousa-Santos et al. 2014b). Furthermore, managed relocations of fish individuals to areas where survival chance is higher is also considered an option. However, such measure needs to be carefully planned to prevent deleterious ecological consequences. So, to maximize operation success, translocations should only be performed within species' historical range and preventing colonization of new landscapes (Olden et al. 2010).

Figure 3. *Iberochondrostoma lusitanicum ex situ* breeding program in the Vasco da Gama Aquarium. Original.

1.1.2. *Iberochondrostoma lusitanicum*: general description

The genus *Iberochondrostoma* (Robalo et al. 2007a) includes the species *Iberochondrostoma almacai* (Coelho et al. 2005), *Iberochondrostostoma lemmingii* (Steindachner 1866), *Iberochondrostoma lusitanicum* (Collares-Pereira 1980), *Iberochondrostoma olisiponense* (Gante et al. 2010) and *Iberochondrostoma oretanum* (Doadrio and Carmona 2003). This genus is endemic to the South and Central Iberian Peninsula, hence the etymology (Robalo et al. 2007a).

Iberochondrostoma lusitanicum (Figure 4) is a Portuguese endemism with restricted geographical range and currently listed as Critically Endangered by the IUCN, protected by national and international legislation (Rogado et al. 2005; Collares-Pereira et al. 2021; IUCN 2021). Populations of this species are present in the Tagus and Sado river basins, as well as in smaller basins such as Barcarena, Caparide, Lizandro, Colares and Samarra and the small coastal lagoon Lagoa de Abufeira between Tagus and Sado (Rogado et al. 2005; Robalo et al. 2007b; Robalo 2008; Faria et al. 2015; Collares-Pereira et al. 2021). Tagus and Sado populations are highly fragmented and significant reductions in distribution range and effective population size have been described (Alves and Coelho 1994; Fluviatilis 2003; Robalo 2008; Sousa et al. 2008). The distinct populations present high molecular variation between them, while within populations the variation is low, revealing the presence of distinct Evolutionary Significant Units – ESU's (Coelho et al. 1997; Mesquita et al. 2001; Robalo et al. 2007b, 2008; Sousa et al. 2008). *I. lusitanicum* presents higher genetic diversity in larger river basins and connected sub-basins (Sousa-Santos et al. 2016). Furthermore, building evidence suggests that the Sado population may represent a distinct species (Robalo et al. 2007b; Sousa-Santos et al. 2019).

Figure 4. *I. lusitanicum* individual collected in February 2018 in Lizandro river, 38.886701˚, −9.298140 ˚. Original.

This small leuciscid (total length: 160 mm) presents a fusiform body with a pigmented lateral line, small head and an arched sub-terminal mouth. Sexual dimorphism is present, with males displaying bigger paired fins and, during reproductive season, nuptial tubercles in the head (Collares-Pereira et al. 2021). Individuals can live up to four years (Collares-Pereira et al. 2021).

This species occurs mostly in small or medium slow water Mediterranean-type streams and individuals are subjected to seasonal fluctuations in water levels, usually being restricted to small river pools in the summer months (Rogado et al., 2005; Robalo et al. 2009). Fishes from this species, similarly to other *Iberochondrostoma* species, have a low capacity to overpass obstacles and present an intermediate tolerance to habitat disturbance (Segurado et al. 2011; Hermoso and Filipe 2021). Swimming performance has been shown to reduce with higher water velocity, especially in smaller animals (Mameri et al. 2020b). Although microhabitat use studies are not available for *I. lusitanicum*, evidence from other *Iberochondrostoma* species suggests that different age classes occupy distinct microhabitats, with larger individuals selecting deeper and sheltered habitats, but congregating in shallower streams in the late summer transition (Santos and Ferreira 2008). Similarly, trophic studies in other species suggest an omnivorous diet composed of zooplankton, plant material, detritus and macroinvertebrates (Collares-Pereira et al. 2021).

Breeding season occurs from March to May (Robalo et al. 2009; Collares-Pereira et al. 2021). When in sympatry, *I. lusitanicum* can hybridize with *Squalius alburnoides* and *I. olisiponense* (Sousa-Santos et al. 2014a; Collares-Pereira et al. 2021). This species is an egg broadcaster and females can perform up to two postures, originating spawning aggregations that can congregate many individuals (Carvalho et al. 2003; Collares-Pereira et al. 2021). Courtship sequences involve one female and one or more males, with males following, touching the female and pressing her body against available objects (e.g., rocks) to stimulate posture (Carvalho et al. 2003). No agonistic behaviours are described between males associated with reproductive sequences, nor outside reproductive season or related to feeding or territoriality (Carvalho et al. 2003; Robalo 2003).

1.1.3. Bacterial pathogens in freshwater fishes

Due to the close association between host and pathogens provided by the aquatic environment, bacterial diseases are a common threat to fishes' homeostasis and survival, both in cultured or free-range conditions. Regarding freshwater fishes, several bacterial genus/species are recognized pathogens, including *Aeromonas salmonicida, Edwardsiella* spp., *Flavobacterium* spp., *Mycobacterium* spp., *Pseudomonas* spp., *Renibacterium salmoninarum*, *Vibrio* spp., *Shewanella putrefaciens,* S*treptococcus iniae* and *Yersinia ruckeri* (Noga 2010; Smith et al. 2019), while *Acinetobacter* spp., *Kocuria rhizophila* and *Stenotrophomonas maltophilia* have emerged as fish pathogens in recent years (Pękala-Safińska 2018).

In freshwater fish, motile aeromonad septicaemia (caused by different mesophilic *Aeromonas* species) is considered one of the most common bacterial diseases (Cipriano 2001), while they can also be pathogenic agents for marine fishes (Noga 2010).

1.1.3.1. *Aeromonas* spp.: general relevance of the genus

The genus *Aeromonas* (from Greek, aer-, gas; -monas: units; gas-producing units) is included in the class Gammaproteobacteria, order Aeromonadales and family *Aeromonadaceae* (Fernández-Bravo and Figueras 2020). Currently, this genus includes 36 species, with many of the newly described species typically limited to a small distribution area (Janda and Abbott 2010; Fernández-Bravo and Figueras 2020).

Members of this genus are Gram-negative straight cells with round ends (0.3–1.0 × 1.0–3.5 µm), coccobacillary to bacillary, oxidase and catalase positive, facultative anaerobes and non-spore forming (Figure 5). They are capable of tolerate increasing concentrations of NaCl up to 5%, survive at pH 5 and their growth temperature can range from 0 to 45 ºC (Parker and Shaw 2011; Stratev et al. 2012; Martin-Carnahan and Joseph 2015; Pessoa et al. 2019; Fernández-Bravo and Figueras 2020).

Traditionally, species from this genus are divided in two groups: mesophilic *Aeromonas*, which are motile isolates that present optimal growth at 35-37 °C; and psychrophilic *Aeromonas*, which are non-motile that present optimal growth at 22-28 °C (Fernández-Bravo and Figueras 2020); however, some non-motile species can harbour flagellin genes and grow at higher temperatures (e.g., *A. salmonicida*; Martin-Carnahan and Joseph 2015). Differences in colony morphology are also noted between the two groups. While psychrophilic *Aeromonas* appear as pin-points within the first 24 h of incubation (22 ºC), mesophilic *Aeromonas* colonies are circular and convex, with 1-3 mm in diameter (35 ºC) (Martin-Carnahan and Joseph 2015; Pessoa et al. 2019).

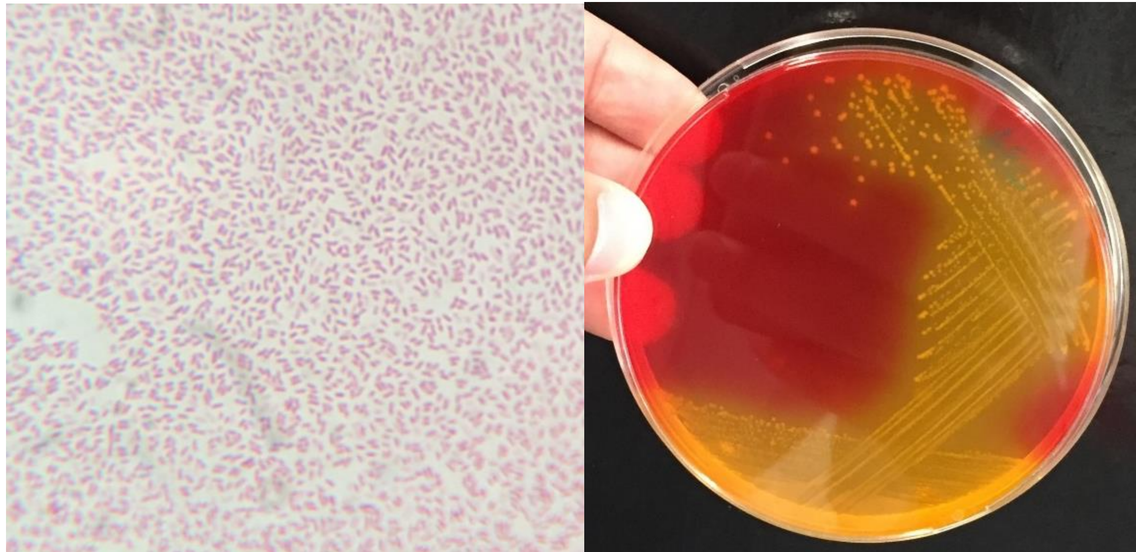

Figure 5. ***Aeromonas*** **sp. morphology observed by microscopic examination (x1,000) with Gram staining (left) and** ***Aeromonas*** **sp. colonies in Glutamate Starch Red Phenol Agar, a selective and differential medium (right).** Original.

Species identification is challenging, with conventional biochemical tests revealing inconsistency due to high phenotypical heterogeneity within some species and constant reclassification within the genus (Janda and Abbott 2010). In fact, rapid identification systems are not accurate in identifying *Aeromonas* species other than the common *A. hydrophila*, *A. caviae* and *A. veronii*, often misidentifying rarer species or misidentifying the genus as *Vibrio* spp. (Janda and Abbott 2010; Martin-Carnahan and Joseph 2015). Due to these challenges, identification based on molecular techniques is still the best option for *Aeromonas* species identification (Tomás 2012; Fernández-Bravo and Figueras 2020). Despite the wide use of 16S rRNA sequencing in bacterial taxonomy, a very high similarity of this fragment observed between different *Aeromonas* species unables the use of this technique as a species discriminatory function (Martin-Carnahan and Joseph 2015). Alternatively, the amplification and sequencing of housekeeping genes, such as *gyrB* and *rpoB*, has proved as the best methodology for species identification and taxonomic classification (Persson et al. 2015; Pessoa et al. 2019).

Aeromonas spp. are ubiquitous to a wide array of ecosystems, although they are more commonly found in aquatic environments, such as surface (fresh, brackish and seawater), underground, potable, bottled, residual, irrigation and waste water (Fernández-Bravo and Figueras 2020), and are especially prevalent in nutrient-rich waters and increase in abundance with environmental temperature (Janda and Abbott 2010; Martin-Carnahan and Joseph 2015). Additionally, *Aeromonas* spp. are frequently isolated from various foods, such as fish,

molluscs, shellfish, beef, pork, poultry, sausages, eggs, milk, cheese and vegetables, as well as in ready-to-eat foods (Stratev et al. 2012), with prevalence limited by temperature, salinity and pH conditions (Fernández-Bravo and Figueras 2020). Similarly, isolation from domestic and wild animals from various taxonomic groups is also described (Janda and Abbott 2010; Parker and Shaw 2011).

1.1.3.2. Mesophilic *Aeromonas* as pathogens

The genus *Aeromonas* have been described as pathogens from a wide range of hosts, including homeothermic and poikilothermic animals. Although initial belief classified these bacteria as opportunistic agents, causing disease only in immunocompromised individuals or after initial infection by other pathogens, current evidence suggests that *Aeromonas* spp. can be primary pathogenic agents in a variety of disease presentations (Martin-Carnahan and Joseph 2015). Furthermore, case occurrence varies according to geographical regions and is intrinsically connected to sanitation and hygiene habits (Fernández-Bravo and Figueras 2020). Another feature of *Aeromonas* infections, and especially mesophilic aeromonads, is its seasonality, with the incidence increasing in summer months (Janda and Abbott 2010).

Considered an animal pathogenic agent since its first isolation from diseased frogs (Sanarelli 1891), *Aeromonas* spp. have been associated with multiple disease presentations in animals including red-leg syndrome in amphibians (Rigney et al. 1978), bronchopneumonia in wild boar (Risco et al. 2013), septicaemia in dogs, septic arthritis in calves, ulcerative stomatitis in snakes and lizards (Gosling 1996), seminal vesiculitis in bulls (Moro et al. 1999), septicaemia in seal (Krovacek et al. 1998) and diarrhoea in livestock (Efuntoye 1995), among others.

Additionally, *Aeromonas* spp. are considered zoonotic agents and emerging pathogens, causing a wide spectrum of diseases in humans and becoming more noticed in Human Medicine as primary pathogens in recent years (Stratev et al. 2012; Fernández-Bravo and Figueras 2020). Generally, disease presentation is mainly related with gastroenteritis, wound and skin infections and septicaemia, with other diseases such as pneumonia, liver abscesses, muscular infections, meningitis, urinary tract infections, joint and bone disease and ocular infections occurring to a lower extent (Parker and Shaw 2011; Pessoa et al. 2019). Most cases in humans are related to *A. hydrophila*, *A. caviae*, *A. dhakensis* and *A. veronii* (Fernández-Bravo and Figueras 2020), and clinical *Aeromonas* pools are considered to be distinct from environmental pools (Martin-Carnahan and Joseph 2015). Ingestion of contaminated foods and water is considered the major route for pathogen acquisition, but aquatic recreational activities or direct contact with water are associated with skin and wound

infections, as well as animal bites (Janda and Abbott 2010). Additionally, the importance of the members of this genus as pathogens is highlighted during natural disaster events, such as tsunamis and hurricanes, with high numbers of *Aeromonas* spp. infections being reported after these events (Parker and Shaw 2011).

Aeromonas spp. have been responsible for several outbreaks in wild and cultured fishes associated with great economic losses (Janda and Abbott 2010). By far, *A. hydrophila* has been the mesophilic species most associated with disease in fishes (Noga 2010), but other mesophilic species have been implicated as fish pathogens, such as *A. allosaccharophila* (Shahi et al. 2014), *A. bestiarum* (Kozińska 2007), *A. caviae* (Wu et al. 2020), *A. jandaei* (Dong et al. 2017), *A. sobria* (Toranzo et al. 1989) and *A. veronii* (Dong et al. 2017). However, retrieving *Aeromonas* spp. from a host is not necessarily associated with disease, as these bacteria can be isolated from healthy hosts (Janda and Abbott 2010).

Depending on the progression of the disease in fish, clinical presentations can vary, but the most common lesions include haemorrhagic fin and tail erosions, cutaneous haemorrhage and skin ulcerations with focal haemorrhage and inflammation possibly extending to the muscle and development of necrotic areas. Acute infections often result in generalized septicaemia. Additional signs include exophthalmia, ascites, visceral petechiation and intestinal and vent haemorrhage and distention. Chronic cases are often characterized by anorexia and skin darkening (Noga 2010; Smith et al. 2019; Figure 6). Disease onset is often correlated with stressful environments (Pessoa et al. 2019) and several risk factors have been identified: higher water temperature, high populational density, water pollution, low dissolved oxygen levels and co-infections (Noga 2010).

Figure 6. *Squalius pyrenaicus* individual displaying haemorrhagic darker areas in the lateral side of the body, in the peduncle and around the vent. Several internal organs were haemorrhagic. The individual died in captivity and anterior kidney bacterial culture and biochemical identification with the API® 20NE system revealed an infection by *Aeromonas* sp. Original.

1.1.3.3. Virulence mechanisms

Aeromonas virulence is considered a complex and multifactorial process (Citterio and Biavasco 2015), resulting from cascades of genetic regulation, often coordinated with environmental alterations (e.g., water temperature oscillations), that lead to the expression of multiple virulence factors (Rasmussen-Ivey et al. 2016a). The expression of these factors acts at different stages, such as tissue adhesion, colonization, immune evasion, proliferation and dispersal in the host, contributing to bacterial pathogenicity (Pessoa et al. 2019). Several genes have been identified, encoding information to produce toxins, structural components, secretion systems and metal-associated proteins (Fernández-Bravo and Figueras 2020). The virulence arsenal of *Aeromonas* spp. include structural components, the production of toxins and other extracellular proteins, secretion systems, iron-binding systems and Quorum Sensing (Janda and Abbott 2010).

Structural components include flagella, pili, the capsule, the S layer and lipopolysaccharides (LPS), which are fundamental for adhesion and initiating colonization (Fernández-Bravo and Figueras 2020). Flagella are responsible for bacterial mobility and, in this genus, a single polar flagellum expressed constitutively and lateral inducible flagella are identified (Citterio and Biavasco 2015). Regulators of lateral flagella expression include surface contact and viscosity (Rasmussen-Ivey et al. 2016a). While the polar flagellum allows mobility in liquid environments, lateral flagella confer mobility in solid surfaces and contribute to biofilm formation and persistence during the infectious process (Pessoa et al. 2019). Pilli are filamentous structures present at the surface and are involved in the adhesion process, biofilm formation, invasion of host cells and DNA transfer (Fernández-Bravo and Figueras 2020). In *Aeromonas*, type IV pilli have been related to pathogenicity and are more common in clinical isolates (Pessoa et al. 2019). The capsule is a structure composed of polysaccharides covering the outer membrane, contributing to resistance to phagocytosis and the complement system and favouring interactions with bacteria and host cells (Tomás 2012). Proteins present in this structure contribute to the adhesion to membrane components of the host cell (Pessoa et al. 2019). The S layer is a surface protein layer that forms the outer part of the bacterial cell envelope (Fernández-Bravo and Figueras 2020). The LPS is composed of the O antigen, a central polysaccharide and lipid A. These components are responsible for the maintenance of the outer membrane and promote a non-specific inflammatory response (Pessoa et al. 2019). Jointly, capsule, O antigen and S layer proteins enable protection mechanisms to evade the host's immune system (Rasmussen-Ivey et al. 2016a).

Much of the pathogenic interaction between *Aeromonas* spp. and host cells is mediated by the extracellular proteins and toxins that the bacterial cell produces and secretes into the extracellular space (Fernández-Bravo and Figueras 2020). These components include

enterotoxins, haemolysins, lipases, proteases and Shiga toxins, among others (Citterio and Biavasco 2015; Rasmussen-Ivey et al. 2016a). Both cytotonic and cytotoxic enterotoxins are known in *Aeromonas* spp., with the later capable of producing haemolysis and inhibiting phagocytosis (Fernández-Bravo and Figueras 2020). Haemolysins promote pore formation in the cell membrane, generating osmotic lysis, for which aerolysin is the most documented one in this genus (Rasmussen-Ivey et al. 2016a; Fernández-Bravo and Figueras 2020). Lipases act as hydrolases on membrane lipids, originating nutrients for the bacterial cell, but also affecting immune system function (Fernández-Bravo and Figueras 2020). *Aeromonas* produce proteases such as metalloproteases, acetylcholinesterase and serine protease, promoting invasion by causing direct damage to the host cells or by proteolytic invasion of toxins, but also disabling the complement system or originating nutrients necessary for self-proliferation (Fernández-Bravo and Figueras 2020). Additionally, Shiga toxin production has been documented in *Aeromonas* (Palma-Martínez et al. 2016), acting on the inactivation of ribosomes of vascular endothelial cells that result in cell death (Parker and Shaw 2011).

Secretion systems are involved in the transport of extracellular proteins and toxins to the extracellular medium or directly into the host cell (Fernández-Bravo and Figueras 2020). Four types of secretion systems (T2SS, T3SS, T4SS and T6SS) have been identified in the genus *Aeromonas* (Pessoa et al. 2019). The T2SS is a widely conserved secretion system and participates in the excretion of a wide array of extracellular proteins (Rasmussen-Ivey et al. 2016a). Functioning as an injectosome, T3SS can introduce proteins harmful to cellular metabolism directly into the host cell cytoplasm. It seems that this system is directly related to pathogenicity (although compensatory secretory mechanisms can exist), as strains mutated for T3SS show lower virulence and occurrence is higher in clinical strains (Rasmussen-Ivey et al. 2016a; Pessoa et al. 2019; Fernández-Bravo and Figueras 2020). The T4SS is the only secretory system capable of transporting DNA besides proteins, revealing an important role in the propagation of virulence genes (Fernández-Bravo and Figueras 2020). The T6SS acts both as an injectosome and a secretory apparatus (Citterio and Biavasco 2015), although it can also play a role in polymicrobial infections by eliminating competing bacteria with antimicrobial pore-forming proteins (Rasmussen-Ivey et al. 2016a; Fernández-Bravo and Figueras 2020).

Metal ions are an important aspect of the host-pathogen interaction. During an infection, host mechanisms try to restrict accessibility of important metals, while bacterial cells compensate with protein production (Pessoa et al. 2019; Fernández-Bravo and Figueras 2020). Iron acquisition is particularly important and can occur via siderophore-dependent or -independent mechanisms (Pessoa et al. 2019). Siderophores, such as enterobactin and amonabactin in the case of *Aeromonas*, are proteins that have affinity with iron ions and

incorporate this metal into the bacterial metabolism (Fernández-Bravo and Figueras 2020). Bacterial outer membrane proteins constitute siderophore-independent mechanisms that bind host-specific iron and extract it from complex protein molecules such as haemoglobin, lactoferrin and transferrin, a function not possible for siderophores (Pessoa et al. 2019).

Biofilm is an additional important virulence mechanism in *Aeromonas* spp. since it confers several advantages such as resistance to antimicrobial agents and adhesion to host tissue. Biofilms are extracellular polymer matrixes composed of proteins, polysaccharides and DNA (Pessoa et al. 2019). In these matrixes, several microorganisms interact with each other and take advantage of the protection this matrix offers, as well the nutrients extracted from the environment (Fernández-Bravo and Figueras 2020). Bacterial intercellular communication in the biofilm is mediated by Quorum Sensing, a chemical signalling pathway controlled by the production of acylated homoserine lactones (AHLS) (Rasmussen-Ivey et al. 2016a). Such signalization between strains allows the microbial community to modulate nutrient uptake, to compete with other microorganisms and to promote defence against phagocytosis (Pessoa et al. 2019). Mutation of signal transduction systems in *Aeromonas* leads to a lower secretion of virulence factors, resulting in attenuation of virulence (Rasmussen-Ivey et al. 2016a).

1.1.4. Conclusion

A vast majority of Iberian leuciscids species display concerning levels of extinction risk. Efforts to identify potential threats to these species have been made, but health aspects have been consistently neglected. The potential of infectious agents in modulating fish survival and, hence, species conservation is considerable. Due to mesophilic *Aeromonas* spp. ubiquity in aquatic environments, the close relationship these organisms can have with fish species and their role as fish pathogens with variable pathogenic potentials, the investigation on the interaction between members of this bacterial genus and threatened Iberian leuciscids is suggested.

Sub-Chapter 1.2. Establishing health surveillance frameworks for threatened Iberian leuciscids

1.2.1. Introduction

Freshwater fish species are experiencing the highest extinction risk among vertebrates in this century (Baumsteiger and Moyle 2017). This is both a reflection and a consequence of the decrease and degradation of suitable freshwater ecosystems observed worldwide (Darwall and Freyhof 2015). While some species present higher tolerance to the disturbances observed at the habitat level (Segurado et al. 2011), others which exhibit specific life traits that predispose them to a higher extinction risk (Kopf et al. 2017; Liu et al. 2017) fail to adapt to these changes and experience significant reductions in their wild stocks. Iberian leuciscids are one example of the last. Although a high level of endemism is observed among leuciscids from the Iberian Peninsula (Sousa-Santos et al. 2019), most of these species face significant extinction risk (Doadrio et al. 2011).

The role that health stressors, like infectious diseases, have in the sustainability of these populations is not clear. Determining risk factors for disease emergence, differences in susceptibility among different species and the geographical distribution of stressors is fundamental for advanced planning and a critical step in controlling and preventing outbreaks that can result in mass mortalities (Maceda-Veiga et al. 2019). The uprising of conditions impacting fish health in the wild is of concern and needs to be addressed as an integrative part of conservation programs. Examples of such conditions include the emergence of pathogenic agents due to climatic alterations (Marcos-López et al. 2010) and exotic species proliferation (Gozlan et al. 2005), or disturbances in the animal's homeostasis due to pollutants accumulation (Molbert et al. 2021).

Despite no studies, so far, have investigated the benefit of conducting health surveillances in the conservation of endangered fish populations, some authors (Berthinussen et al. 2021; Kophamel et al. 2021) stress the importance of these frameworks as early warning systems regarding emerging pathogens. Furthermore, it is suggested that the information gathered in surveillances can be used to apply measures that minimize the risk that specific agents might pose on imperilled populations. Hence, the development and standardization of health surveillance schemes that can be applied to fish populations facing extinction risk, in particular to threatened Iberian leuciscids, are required.

1.2.2. Health assessments are missing in threatened Iberian leuciscids

Despite several efforts in identifying and recording the threats to which endangered Iberian leuciscids are exposed to (Maceda-Veiga 2013), evaluations of the general health status of these populations, as well as the study of infectious agents' prevalence and impact, are limited.

Investigations on the health status of these species have included techniques such as gill histopathology for accessing changes related to water quality (Pereira et al. 2013; Cortes et al. 2016; Santos et al. 2019; Santos et al. 2021), oxidative stress analysis (Pereira et al. 2013; Cortes et al. 2016), nutritional condition or biomass assessment (Nicola et al. 2010; Aparicio et al. 2011; Cortes et al. 2016; Maceda-Veiga et al. 2017; Ramos-Merchante and Prenda, 2018) and the use of indexes that take into consideration visible abnormalities as a consequence of habitat degradation (Matono et al. 2009; Ramos-Merchante and Prenda, 2018).

Regarding pathogenic agents, prevalence studies have focused mainly on parasitic infections, with descriptions including fungal infections as well (Aller-Gancedo et al. 2016) (Table 2; a more extensive description can be found in Supplementary Table S1, Annex I).

Table 2. Summary of parasitic and fungal diseases' prevalence studies in threatened Iberian leuciscids.

Agents	Reference
Parasites	
Mussels	Illán 2012; Reis et al. 2014; Teixeira et al. 2018; Dias et al. 2020
Copepods	Perez-Bote 2000; Illán 2012; Sánchez-Hernández 2017
Trematodes	Simon Vicente and Ramajo Martin 1971; Simon Vicente et al. 1973; Simon Vicente 1975; Bueno 1980; Álvarez-Pellitero et al. 1981; Illán 2012; Illán et al. 2013; Čermáková et al. 2018; Benovics et al. 2020; Benovics et al. 2021
Cestodes	Martinez Gomes 1970; Bueno 1980
Nematodes	Bueno 1980
Myxosporeans	Gonzalez-Lanza and Álvarez-Pellitero 1985; Illán 2012; Rocha et al. 2019
Ciliates	Illán 2012
Fishes	Silva et al. 2013
Fungi	
Oomycetes	Aller-Gancedo et al. 2016

Unfortunately, due to the nature of the infectious agent location in the host or the need of organ sampling for health evaluations, several studies have relied on animal euthanasia to perform health investigations. Although understandable in the context in which most of the studies were performed, this approach is unsustainable for threatened populations

experiencing severe declines in their natural stocks. Furthermore, the focus of many of these studies has been to describe the prevalence of infectious agents in these species, rather than evaluating the impact such agents have at the populational and species-level. However, this is not always the case, with some studies investigating infection intensity, lesion patterns, individual condition and risk factors associated with disease onset (Illán 2012; Silva et al. 2013; Aller-Gancedo et al. 2016; Sánchez-Hernández 2017; Maceda-Veiga 2019; Maceda-Veiga et al. 2019; Grilo et al. 2021).

With such investigations, the link between host-pathogen interactions and the effects of environmental drivers and external stressors to the habitats has been clarified, reinforcing the idea that several disruptors are key in the proliferation and impact of pathogens in natural habitats, as well as in the general homeostasis of fishes.

1.2.3. Environmental alterations as optimal grounds for health imbalances

The close relationship and the interdependence fish experience with their aquatic surrounding make this animal group highly susceptible to environmental changes in their ecosystems.

Several environmental parameters have the potential to create disruptions in fish health. Namely, factors such as oscillations in water's temperature and pH, oxygen depletion, stress, confinement, high levels of organic matter and exposure to xenobiotic chemicals and biotoxins have all been implicated with the development of conditions that impact the host's homeostasis (Plumb et al. 1976; Noga 2000; Udomkusonsri and Noga 2005; Murawski et al. 2014; Granneman et al. 2017; Lamb et al. 2018; Vajargah et al. 2018).

This environmental effect is of particular importance in areas exposed to severe climate-imposed alterations in the habitat, such as those observed in Mediterranean-type streams of the Iberian Peninsula. Several threatened fish species occur in these intermittent streams, located in the southern part of the peninsula, and are exposed to typical cyclical shifts in water levels as a consequence of droughts occurring in the summer and floods in the winter. During the dry season, significant flow reduction often results in the fragmentation of the river, restricting both the fish's habitat as well as their ability to move and find more suitable environments (Sousa-Santos et al. 2016). Such situation will result in a landscape composed of more or less disconnected pools that often congregate a high number of individuals (Sousa-Santos et al. 2016). This increased density effect will have obvious consequences in water quality and resources' availability. Higher animal numbers quickly contribute to water quality's deterioration – since water renovation is low or even null, excretion products accumulate, and higher water temperatures and lower dissolved oxygen levels are experienced (Magoulick and

Kobza 2003). Additionally, intra- and inter-specific competition increase due to limited prey items and space, promoting contact between animals (Vedia et al. 2019).

What these conditions generate is often one of the worst disturbances for an animal's general fitness – i.e., stress. Also, and regarding pathogenic agents, several reports evidence how these agents benefit from disturbed environments with higher temperatures and pollution, resulting in increased prevalence and transmission rates (Maceda-Veiga et al. 2009; Sánchez-Hernández 2017; Maceda-Veiga et al. 2019; Grilo et al. 2021). An additional concern is the role of exotic and invasive species and their potential contribution as vectors of pathogenic agents. Such individuals can either introduce new pathogens in naïve populations or promote the interchange of different strains across habitats, a situation that can result in detrimental effects for fishes' health or, ultimately, generate mass mortality events (Maceda-Veiga et al. 2009; Gozlan et al. 2010). So, the cumulative effects of these stressors will have profound effects on the host, either directly (e.g., structural lesions related to exposure to xenobiotics and pathogenic agents) or indirectly (e.g., depressed immune function and higher susceptibility to opportunistic infections) (Ventura and Grizzle 1987; Austin 1998; Noga 2000; Law 2001; Marques et al. 2016). This is particularly important in younger life stages, such as juvenile fishes, who naturally experience an impaired immune function due to immaturity (Cornet et al. 2020).

To accommodate a proper monitoring of the changes in the health status at the populational level of these imperilled fish species, standardized health surveillance schemes should be implemented.

1.2.4. Health surveillance frameworks as valuable conservation tools

The establishment of health surveillance frameworks in threatened fish populations presents several advantages. At an initial stage, such schemes produce basal information on the physiological health status of a population that can be later used to identify disease states by comparison with normal observations (Kophamel et al. 2021). In this sense, standardized health assessments assume a vital role in the identification of disease risk and associated decline of wild fish populations, especially in the identification of the underlying causes inducing disease. The knowledge of the prevalence of nutritional deficiencies related to starvation, diseases caused by infectious agents or exposure to xenobiotics with detrimental effects, or, at a larger scale, to understand the impact that alterations in the ecosystem (e.g., environmental parameters fluctuations) have in fish health, is essential for strategic planning of adequate human interventions regarding wildlife conservation (IUCN 2017). Furthermore, and with a broader recognition of the One Health concept and the interaction between distinct

populations and habitats, the routine monitoring of infectious agents or diseases in wild fishes assists in the early detection of pathogen presence and in the prevention of transmission to domestic animal and human populations (Stitt et al. 2007; Woods et al. 2019). Past efforts in establishing health surveillance in threatened species as established by the International Union for Conservation of Nature Red List are still limited and future efforts should be made for such species (Kophamel et al. 2021).

Several methodologies can be applied to evaluate the health status of a wild animal/population. These include physical exams to conclude data regarding morphometry or behaviour of the animal, the investigation of infectious diseases prevalence and impact and the use of animal samples to perform further investigations (e.g., blood analysis, faecal analysis, determination of xenobiotic levels, genetic investigations). The extent to which each methodology was employed in prior health assessments is variable (Kophamel et al. 2021). Selection of which methodology will be employed when establishing a health surveillance in fishes will vary according to the needs of the surveillance scheme and depend on the availability of funds and appropriate methods calibrated for these species, as well as the possibility of employing such methods when working with wild fishes. Although some methods (e.g., blood analysis) might provide valuable information regarding individual homeostasis, the challenges related to the needs of collecting the sample, the stress that is produced in the animal that can impact its current or future survival and the difficulties associated with field work need to be considered and re-evaluated regarding the information other less challenging methods can offer.

Another important consideration is related to sample size. While robust statistical significance should be pursued to produce diagnostic parameters that can be reliable and representative of the population (Kophamel et al. 2021), when considering threatened fish species, sampling can be highly unpredictable, rapidly fluctuating with environmental shifts due to climatic or anthropogenic factors. In that situation, sampling fewer individuals should be viewed as an important tool that can provide indications of the general health status of the population. A final consideration should be made regarding the use of Citizen Science as an important method to collect data on health impacts in fish populations. This methodology is particularly relevant in situations of limited funding and can help to produce data on morbidity and mortality in wild animals that can focus research efforts on a particular population/area (Lawson et al. 2015).

A detailed conceptual framework for preparing and establishing health assessments across a wide taxonomic array was discussed in Kophamel et al. (2021) and its consultation prior to the implementation of health surveillance schemes is advised. Health surveillance can provide vital information that should be used in conservation management, taking into

consideration the vulnerability status of some populations/species to health impairments, and counteracting on them to assure the sustainability of biodiversity (Kophamel et al. 2021). Additionally, active surveillances can identify emerging pathogens that may affect human and animal health, and the prophylactic control of these agents can present socioeconomic advantages, such as the decrease of costs associated with the impact of emerging diseases in human and domestic animals, the prevention of spill-over events and to ensure ecosystem protection and function (Grogan et al. 2014). Ultimately, preventing diseases in wild fish populations by establishing early detection systems and appropriate control measures has a feedback effect – ecosystem stabilization will result in a lower emergence of new pathogens (Pongsiri et al. 2009).

1.2.5. Conclusion

Current concerns and recommendations expressed for threatened Iberian fishes have a broader application, since such considerations can be applied worldwide to other wild fish populations facing extinction and regarding whom a standardized health surveillance has not been performed yet. Health assessments should be an essential part of any conservation plan, since neglecting the important knowledge these interventions can disclose is to disregard the protection of threatened fish species as a holistic plan, hampering the future success of fish conservation science.

Sub-Chapter 1.3. The potential of *Aeromonas* spp. from wildlife as antimicrobial resistance indicators in aquatic environments

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1.3.1. Abstract

Worldwide emergence of bacterial strains resistant to multiple classes of antimicrobials and the increase incidence of infections caused by bacteria resistant to last-resource antibiotics is now a common problematic and likely to increase in coming years. Surveillance of important resistant clones and associated mobile genetic elements is essential for decision-making in terms of mitigation measures to be applied for the prevention of such infections.

However, the role of natural environments, and especially aquatic ecosystems that display optimal grounds for antimicrobial resistance (AMR) development and dissemination, as important components of the AMR cycle has been disregarded until recent years. It is now widely accepted that resistant strains and resistance determinants are disseminated in the aquatic environment and that resistance profiles are mainly shaped by anthropogenic environmental contamination.

In light of the One Health concept, developing strategies for the monitoring of AMR prevalence and dissemination and unravelling critical points of action are required. Therefore, bacterial indicators and targeted vectors assume special importance in surveillance schemes.

Aeromonas spp., a ubiquitous bacterial genus with importance as human, animal and food pathogens, are common in aquatic environments. Environmental isolates belonging to this genus often display acquired resistance determinants and their use as indicators to survey water quality and sewage pollution has been accepted. Wild animal species are also of highly importance in this context. They constitute potential AMR reservoirs in natural environments, as they actively participate in the dissemination of resistant bacteria and resistance determinants across habitats.

Characterizing *Aeromonas* spp. retrieved from wildlife represents a potential aid for surveillance programs aiming at unravelling the intricate mechanisms of AMR evolution and dispersal in natural aquatic environments, as well as understanding possible consequences for human and animal health.

1.3.2. Introduction

AMR evolution and dissemination are intrinsically connected to genetic determinants encoding for resistance against a variable range of antimicrobial compounds (Paulson et al. 2016). This phenomenon occurs naturally as a consequence of microbial competition; hence, such determinants can be present in natural environments as a result of point mutations (Caniça et al. 2015; Fletcher 2015). However, selective pressure due to frequent and widespread antibiotic use and the introduction of antibiotic residues, antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) in natural habitats by anthropogenic activities has significantly influenced resistance profiles of environmental microbial communities at a higher level than naturally occurring antibiotics and genes (Goulas et al. 2018). Traditionally, limited attention has been given to the role that natural ecosystems and environmental factors have on the dissemination of AMR (Fletcher 2015), as surveillance and control efforts focused on clinical settings (Ekwanzala et al. 2018). In recent years, natural aquatic environments have received growing attention in terms of characterizing the prevalence of antimicrobial compounds, ARGs and ARB, being increasingly more acknowledged as reservoirs of AMR and a potential threat for public health (Chen et al. 2018). Despite the increase of investigations in this field, the impact of entry points on AMR levels in the environment is not fully understood (Bueno et al. 2018).

The growing emergence of strains resistant to multiple antimicrobial compounds constitutes a public health problem. Understanding these strains' spatial distribution and factors promoting resistance is crucial. Due to the complexity of ecosystems, control in natural environments can be difficult once resistant microbial communities are established. Surveillance programs aiming at unravelling ARB prevalence and points of origin can contribute to countering the persistence of high levels of ARB and ARGs in the environment (Alexander et al. 2015).

In its last report on AMR surveillance, the World Health Organization (2015) pointed out the lack of methodology guidelines to survey AMR at various geographical areas, as well as gaps in the established surveillance programs. Broad-scale sampling strategies are essential to fully unravel the aquatic environmental resistome and its consequences for public health (Hu et al. 2017). Microbiota present in wild species may appear as important indicators

to study AMR prevalence and dissemination in natural environments and constitute a potential aid for monitoring programs. In this review, we explore general concepts regarding AMR origin and dissemination in aquatic ecosystems, as well as its detection and relevance under a One Health perspective, and discuss the characteristics and potential framework that can make *Aeromonas* spp., an ubiquitous aquatic bacterial genus, and wild animal species interesting sentinels to be used under surveillance schemes of AMR in aquatic environments.

1.3.3. From land to water: aquatic environments as reservoirs of AMR

Our knowledge on the role of aquatic ecosystems as reservoirs and disseminators of AMR increased in later years (Bueno et al. 2018). Nowadays, ARBs, ARGs, antimicrobial residues and other pollutants are widely accepted as emerging contaminants of these settings with a significant influence in driving and maintaining AMR cycles at the aquatic level (Ahmed et al. 2018; De la Cruz Barrón et al. 2018).

Aquatic habitats display ideal characteristics both for horizontal gene transfer (HGT) and for the establishment of environmental gene and bacterial pools (Bhattacharyya et al. 2019). HGT is an important mechanism for the transmission of ARGs at the intra- and inter-species level (Cairns et al. 2018); especially regarding the transmission of genetic information from environmental non-pathogenic bacteria to potential pathogenic bacteria of clinical interest (Paulson et al. 2016). Due to high throughput metagenomics techniques, the level of exchange of genetic determinants between ARB of environmental and clinical origins has become more noticeable in recent years (Ekwanzala et al. 2018).

Anthropogenic influence is considered to be the most relevant origin of AMR in natural aquatic environments, and entrance in the ecosystem can occur through several routes (Figure 7) (Berendonk et al. 2015; Le Page et al. 2017; Shao et al. 2018). ARB present in the human digestive tract are of high importance in this context. These strains commonly enter aquatic streams trough sewage effluents and sludge. Although many efforts have been made in order to effectively eliminate ARB and ARGs from human effluents, wastewater treatment plants are still incapable of guaranteeing their total elimination (depending on the bacterial genus and ARGs) (Bergeron et al. 2015; Adegoke et al. 2018; Jäger et al. 2018). Similarly, after treatment in wastewater plants, antibiotics have been found to either retain their total original configuration or to form conjugations, and their release may have potential detrimental consequences for the environment (Adegoke et al. 2018). Such compounds, normally present at sub-inhibitory concentrations, give selective advantage to resistant bacterial communities to emerge and proliferate (Alexander et al. 2015). Similar consequences can occur from runoff discharges originating from fertilized agricultural fields and animal production manure

(Paulson et al. 2016). However, in some situations, ARGs abundance in waste originating from farms is higher than in waste water effluents (Rowe et al. 2016).

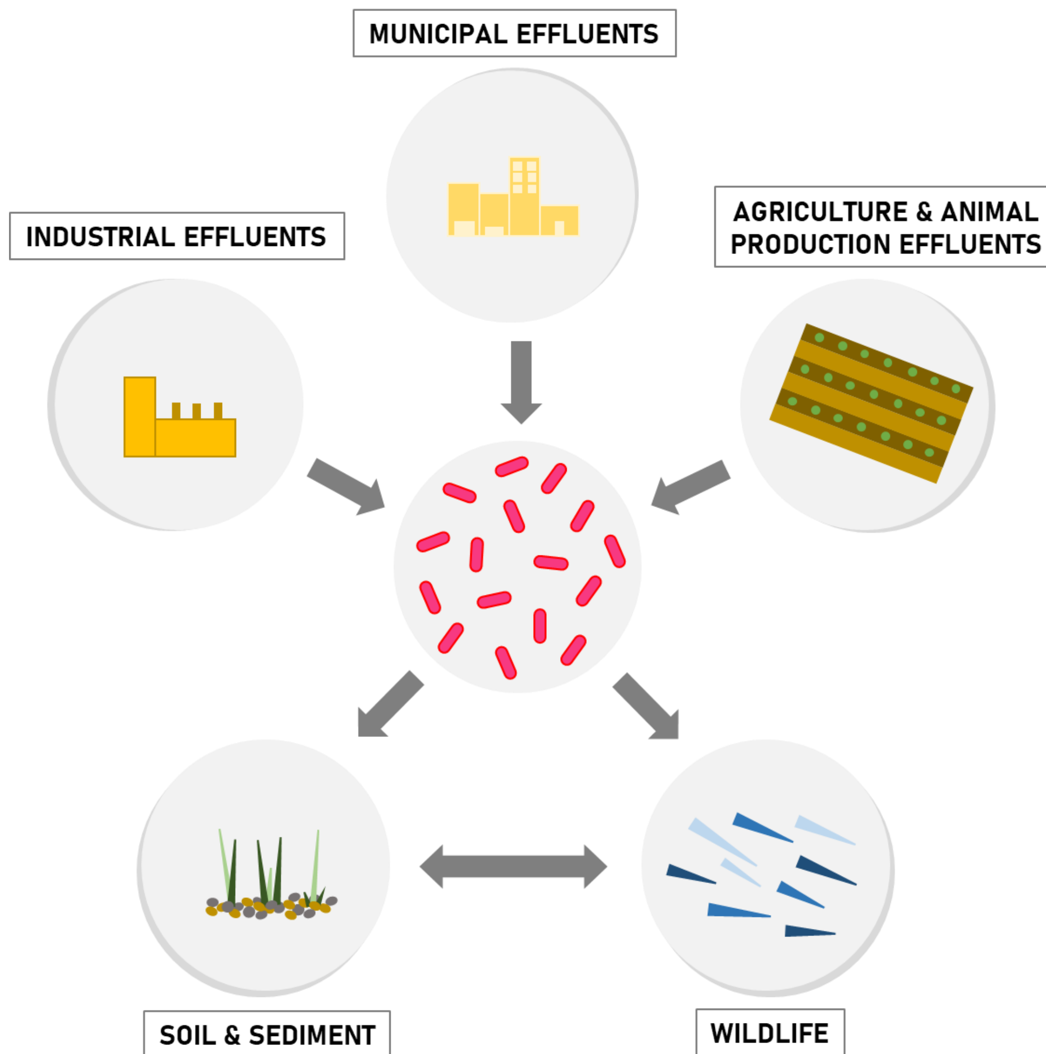

Figure 7. Main anthropogenic origin routes of antimicrobial resistance determinants into natural aquatic environments and associated reservoirs.

Environmental bacteria from aquatic ecosystems can allow ARGs discharged via multiple anthropogenic origins to persist and integrate the genome of potential pathogenic bacteria, potentially being transmitted to humans via multiple sources (Czekalski et al. 2015). Indeed, there are multiple entry points from which ARGs and ARB released into the aquatic environment may contact with humans. Besides drinking water from contaminated areas, aquatic recreational activities and ingestion of crops irrigated with wastewater are also established contact routes (Berglund 2015).

AMR cycles in aquatic ecosystems have important consequences for public health and need to be addressed under the One Health initiative, in order to try to tackle the problem simultaneously in human, veterinary and environmental health. Water environments can contribute to the transformation of localized AMR niches into global intricate networks of AMR dissemination (McEwen and Collignon 2018), either due to water flow, transformation of waterborne bacteria into airborne, wildlife migration or anthropogenic dissemination of vectors (Taylor et al. 2011).

Society development and sustainability is highly correlated with water availability, relying on aquatic environments for several activities, e.g., drinking, agriculture irrigation, livestock production and leisure activities. Either by direct contact with water or via food consumption, humans and animals have the potential to be contaminated with pathogenic and environmental ARB, as well as with other AMR determinants. Host gut environment is then an ideal setting for HGT, allowing the share of AMR determinants between different bacterial genera and the establishment of new reservoir species (Taylor et al. 2011).

The identification of the intricate network of environmental pathways that can contribute to disseminate AMR into aquatic environments and later contaminate different ecological drivers is essential for mitigation measures' proposal. Surveillance schemes using sentinel species such as wildlife, instead of water samples, have the potential to enhance this process by providing complex ecological interactions, which will contribute to a more realistic scenario of AMR in aquatic ecosystems.

1.3.4. Wildlife: reservoirs/ disseminators/sentinels of AMR

As a consequence of AMR dissemination into natural environments, such as aquatic ecosystems, wildlife of different ecological niches can become AMR reservoirs, as shown by increasing evidence of the presence of ARB and ARGs in these species (Carroll et al. 2015). Scientific community is now aware that these species are not only reservoirs for AMR, but they also participate in natural cycles of AMR dissemination (Hu et al. 2017). In this context, wild animal species are optimal sentinels for AMR prevalence and dispersal at a multi-habitat scale.

As a result of habitat fragmentation and degradation due to global human population increase and land use, direct and indirect contact between humans, domestic animals and wildlife are enhanced and are becoming more frequent. Such proximity establishes interaction bridges that can result in ARB and ARGs sharing (Arnold et al. 2016). One obvious example relates to the loss of wetlands and association of wild species with artificial systems such as waste water treatment plants, which are important sources of ARB and ARGs.

Detection of resistant isolates or resistance determinants in wildlife has become common. A compilation of AMR prevalence studies in wildlife, exposing this situation, is reviewed in Vittecoq et al. (2016).

The anthropogenic action in wild animals at this level has as a consequence the establishment of these species as natural reservoirs of AMR, presenting resistance profiles and determinants commonly associated with human settings (Caniça et al. 2015).

Wild animals using resources in ecosystems in close contact with human settings are increasingly more exposed to ARB and ARGs. Effluent and runoff contamination of aquatic ecosystems constitute critical contact points to aquatic wildlife (Arnold et al. 2016). After their acquisition, wild fauna plays an important role on the spread of these determinants across different natural settings (Hu et al. 2017). This is especially important in migratory species that can travel long distances, connecting environments from distinct geographical areas with different microbial landscapes and being exposed to distinct AMR patterns (Dolejska and Papagiannitsis 2018). Also, wild species have also the potential to introduce ARB and ARGs in pristine environments that are not exposed to anthropogenic pollution.

Ecological traits can influence the risk of acquisition and dissemination of AMR. For example, Vittecoq et al. (2016) concluded that species displaying omnivorous and carnivorous regimens are at higher risk of acquiring and disseminating ARB and ARGs. Therefore, evaluating the role of wildlife in AMR and disease modelling can be complicated by their ecological and behavioral characteristics. Factors that need to be accounted for include the disturbance level due to human land use, life-history traits and seasonality (Hassell et al. 2017).

Identifying ARB and ARGs in wildlife is relatively simple, comparing with the detection of antimicrobial compounds in the environment. Wild species can highly contribute as sentinel species for the evaluation of environmental pollution of anthropogenic origin and AMR dissemination (Paulson et al. 2016). In comparison with water samples, wild animals have the advantage to be colonized during longer timespans and to display microbial communities with higher relative abundance and concentration. Bighiu et al. (2019) tested ARB concentration in both water samples and zebra mussels (*Dreissena polymorpha*) and found that the latter had significantly higher concentrations and displayed ARB for a longer period.

Although the impact of ARB and ARGs is thought to be negligible per se in wildlife, disturbance of host-microbiota interactions might have profound consequences for animals' homeostasis since this interaction has effects at the immune system, digestion and behavioural levels. Surveillance of microbiome associated with wildlife can constitute an important indicator tool for evaluating host's health (Trevelline et al. 2019). Additionally,

monitoring the prevalence of AMR in wild species is a useful technique to evaluate anthropogenic pressure and pollution in aquatic environments – with consequences for wild species conservation and for ecosystem health (Foti et al. 2018).

Choosing bacterial indicators to be used in wildlife AMR surveillance schemes relies on the assumption that result comparison between worldwide investigations can be performed. For this, the bacterial indicator needs to occur in broad distribution across different aquatic habitats, as well as in different taxonomic groups. Among the bacterial genus commonly associated with aquatic environments, *Aeromonas* spp. are widely recognized to have a broad host range, being able to colonize and cause illness in both poikilothermic and homeothermic hosts (AbuElala et al. 2015; Carnelli et al. 2017). Their prevalence and susceptibility patterns have been described in different aquatic organisms, like fish (Emond-Rheault et al. 2015), mammal (Lim et al. 2019), avian (Cardoso et al. 2018), reptile (Ariel et al. 2017), amphibian (Hacioglu and Tosunoglu 2014) and mollusc species (Maravić et al. 2013), confirming their potential as bacterial indicators of AMR in aquatic environments.

1.3.5. *Aeromonas* spp. potential as AMR indicators

Aeromonads are gram-negative bacteria ubiquitary in aquatic environments, enhancing their contact opportunities with humans and animals (Janda and Abbott 2010). The genus *Aeromonas* has been isolated from a variety of settings, such as freshwater and marine habitats, groundwater, wastewater, sewage and drinking water (Baron et al. 2017; Igbinosa et al. 2017; Dias et al. 2018). Pathogenicity seems to be variable between species (Ghatak et al. 2016). However, the presence of *Aeromonas* spp. in a host is not mandatorily associated with disease, as these bacteria is frequently isolated from healthy hosts. Such remark is important to understand the role that multiple hosts play as reservoirs of *Aeromonas* spp., as well as their participation in the exchange dynamics of ARGs between clinical and environmental pools of *Aeromonas* spp. (Janda and Abbott 2010).

The development of guidelines for antimicrobial susceptibility testing of *Aeromonas* isolates is still in need of much improvement, despite recent updates have been published. Current established methods are restricted to a limited number of species (*A. hydrophila*, *A. caviae*, *A. veronii* bv. sobria, A. salmonicida), and are often restrictive in terms of antimicrobial compounds, as observed in both the CLSI and EUCAST guidelines (CLSI 2014, 2015; EUCAST 2020). Available breakpoints have derived from clinical criteria. However, several issues make it questionable to perform extrapolations of susceptibility category from clinical to environmental isolates (Alexander et al. 2015). The number of samples needed and the standard categorization system based on the likelihood of therapeutic failure may jeopardize

the safe use of established guidelines for environmental isolates. Recent studies are aiming at developing alternative and more reliable interpretation guidelines, such as using epidemiological cut-off values separating wild-type from non-wild-type (i.e., with acquired resistance) populations. One advantage of this method is to be epidemiological based and its informative value will likely increase together with the number of isolates sampled from environmental origins (in contrast with the current predominance of clinical ones) (Berendonk et al. 2015). Also, the monitoring of AMR in isolates from the genus *Aeromonas* in different geographical areas would benefit from the establishment of standard criteria in order to perform safe comparisons between distinct sampling areas (Baron et al. 2017).

Resistance to antimicrobials in this genus is normally chromosomally mediated (Anandan et al. 2017). Aeromonads are known to harbour gene cassettes, genomic islands, class 1 integrons, plasmids, IS elements and transposons (Patil et al. 2016). It is currently accepted that aeromonads present gene pools which renders the environmental reservoirs for AMR, and that gene diversity allows them to form dissemination networks with other environmental bacteria and potential pathogens. The dissemination of resistance determinants to humans and animals may eventually occur following specific actions, e.g., via consumption of contaminated food or drinking water (Piotrowska and Popowska 2015; Chenia 2016).

Surveillance programs have focused in this genus, alone or in combination with other bacterial indicators, to assess AMR prevalence at the environmental level, for example by including samples from estuarine environments (Chaix et al. 2017), river water (Li et al. 2015; Igbinosa et al. 2017) and hospital and municipal effluents (Varela et al. 2016). Although results vary greatly according to isolate's origin, increasing resistances to aminoglycosides, β-lactams, quinolones and tetracyclines have been recorded (Cardoso et al. 2018). Additionally, building evidence demonstrates the role of wastewater treatment plants in the introduction of resistant strains of *Aeromonas* spp. into freshwater streams (Harnisz and Korzeniewska 2018).

Investigation of *Aeromonas* spp. in aquatic wildlife has already proven successful in unraveling important surveillance questions of AMR in aquatic ecosystems; like the role of migratory species in dissemination across environments (Laviad-Shitrit et al. 2018), AMR contact points between environment and human populations (Maravić et al. 2013), the determination of reservoir status for clinical relevant AMR determinants (Morris et al. 2011; Lim et al. 2019), and the discovery of novel AMR genetic assemblages (Emond-Rheault et al. 2015). Establishing frameworks for AMR investigation using *Aeromonas* spp. from wildlife is key for future comparison across research groups.

1.3.6. Proposing *Aeromonas* spp. from wildlife as bacterial indicators under aquatic environmental frameworks for AMR surveillance

Aquatic environmental frameworks for AMR evaluation need to take into consideration natural constraints characteristic to these habitats. Assessing AMR in aquatic ecosystems and the related environmental risks can be problematic. First, naturally occurring ARGs are present in the environment, which can limit conclusions from direct detection (Fletcher 2015). Second, the natural complexity of aquatic ecosystems difficult the evaluation of the impact of a determined resistance mechanism (conferred by a specific genetic determinant) on the outcome of their interaction with bacterial communities and future hosts (e.g. humans) (De la Cruz Barrón et al. 2018). Different environmental factors, such as environmental physical and chemical characteristics, and bacteria stress response, adaptation and phenotypic diversity; have the potential to regulate selective pressure from resistance determinants and antimicrobial compounds, (Berendonk et al. 2015).

Much of our knowledge on the presence of ARB and ARGs at the aquatic level has been based on occurrence-based studies (De la Cruz Barrón et al. 2018). Recent investigations are increasingly using molecular tools, rather than culture-based methods, in order to better evaluate the real prevalence of AMR in aquatic environments, its epidemiological relevance and to identify the links between anthropogenic effluents and aquatic contaminations (Czekalski et al. 2015). Metagenomic analysis is gaining relevance in environmental surveys since it allows a broader investigation and comprehension of the epidemiological scenario of aquatic environments regarding ARB and ARGs (Garner et al. 2017; Chu et al. 2018; Ju et al. 2018). With these tools, a global profile of microbial communities and their functional composition can be achieved (Port et al. 2014).

Standard global surveys are essential for the characterization and quantification of AMR in the aquatic environment and to establish the real consequences of environmental contamination in the proliferation and dissemination of AMR (Berendonk et al. 2015). Additionally, systemized surveillance of broad geographical areas has the potential to disclosure hot-spots for AMR prevalence and factors influencing it, such as human activity association and water quality parameters (Liu et al. 2018).

Aeromonas spp. display different traits that make them suitable organisms to evaluate the prevalence, development and dissemination of AMR in aquatic ecosystems. As previously mentioned, they are ubiquitous in these settings, in which they are able to persist for long periods of times (Adamczuk and Dziewit 2017). Additionally, the prevalence and dissemination potential of *Aeromonas* spp. in settings with relevance to the One Health approach (humans, animals and the environment), as well as the fact that they are responsible for infections in both humans and animals, assures genus representativeness at the epidemiological level

(Chaix et al. 2017). Finally, it has been shown that they can possess and acquire different mobile genetic elements that can contribute to the dissemination of AMR, not only to environmental bacteria, but also to clinically important species (Baron et al. 2017).

Aeromonas spp. present advantages over more traditional indicator bacteria in water. Although *Escherichia coli* is commonly used to monitor AMR both in human and veterinary medicine, and it is traditionally used as a bacterial indicator at the aquatic level, its isolation from the aquatic environment is not consistent across different streams (Usui et al. 2016). In situations where faecal coliforms are suspected to be absent, *Aeromonas* spp. can be used as indicator species (Berendonk et al. 2015). Additionally, since *Aeromonas* spp. are often found in environments with high concentrations of organic carbon, they are believed to be good indicators for water quality and pollution (Igbinosa et al. 2017). Aeromonads, especially due to their prevalence in sewage waters, meet the criteria required for bacterial indicators, not only in terms of AMR investigation, but also to investigate water quality and pollution, due to the presence of metals and other pollutants (Yang et al. 2019).

The role that aquatic wild animal species can have in environmental surveillance frameworks is well established and sample collection originating from them should be encouraged. Globally, recruiting wildlife hospital staff and wildlife projects' team members to participate in AMR epidemiological studies can increase the sampling size and areas, hence increasing the number of investigated locations and results' significance. Ideally, wild species implicated in AMR dissemination at a larger scale could be more easily identified, improving the implementation of control measures for acquisition of ARB and ARGs (Arnold et al. 2016). However, the role of species with site-fidelity is of particular interest to unravel local dynamics and risk factors (Furness et al. 2017).

There is a lack of investigations that compare spatial distribution and risk factors for the prevalence of ARB and ARGs in wildlife. This acquires special relevance in areas where AMR input is well characterized and AMR dissemination can be followed. Fine-scale movement studies would be valuable in order to fully understand AMR dissemination in wildlife, since it would represent a closer approximation to reality than one-point sampling surveys (Arnold et al. 2016).

Using *Aeromonas* spp. from wildlife as AMR bacterial indicators under environmental frameworks should be interpreted as cumulative knowledge in a multi-scale action plan, rather than a unique solution. For this matter, surveillance schemes simultaneously investigating other established bacterial indicators in water samples – faecal coliforms, enterococci and *Pseudomonas* spp. – are an important advantage.

1.3.7. Conclusion

Mitigation measures to control AMR introduction and spread in natural environments must follow the same strategies used for other forms of environmental pollution. Bottom line, antimicrobial use control at the human and veterinary levels, strict regulation application in terms of waste management and disposal, and technological development of AMR elimination strategies in sewage are essential to reverse and recover from this worldwide problematic (Paulson et al. 2016).

Some crucial knowledge is still missing for the proper management of environmental AMR, namely the contribution proportion of ARB, ARGs and antimicrobial compounds from different sources into aquatic environments presence; the risk to human and animal health associated with contact with environmental ARB and ARGs; the environmental mechanisms that contribute to resistance evolution; and the response of environmental AMR to human evolution at the technological and social levels (Larsson et al. 2018).

Members of the genus *Aeromonas* have been investigated in order to detect antimicrobial resistance determinants, as well as sewage pollution, in aquatic environments. Aeromonads have the potential to be used at a global scale as a fundamental part of surveillance programs in natural environments. Wildlife can play an important role in the AMR dissemination cycle and, thus, surveillance programs need to take into consideration its microbiota for their strategic role as indicators of AMR in natural environments. By doing so, better understanding of impacts for human, domestic animal, wildlife and environmental health can be obtained.

The characterization of *Aeromonas* spp. collected from wildlife using standardized approaches has the potential to allow to survey AMR in aquatic environments and their associated communities around the globe, contributing to AMR dynamics signalling, natural habitats' anthropogenic impact evaluation and mitigation implementation response measuring.

Sub-chapter 1.4. Objectives

The main goal of this PhD thesis was to investigate the prevalence and dynamics of potential pathogenic agents in threatened Iberian leuciscids, both in a perspective of the impact for the conservation of this imperilled group, as well as the possible existence of antimicrobial resistance and virulence reservoirs of concern in the One Health context harboured in wild populations.

In order to achieve the central goal of this thesis, both a pathogen and a host model were selected based on the following criteria:

Pathogen

- Common occurrence in freshwater fishes and known impact in wild populations;
- Prevalence dynamics and effects in the host modulated by environmental variables and degree of habitat disturbance;
- Potential pathogens implicated in historical lesion description detected in fish census conducted with threatened Iberian leuciscids;

Host

- Threatened conservation status as determined by the IUCN;
- Integration in the Portuguese *ex situ* breeding program for endemic leuciscids;
- Distribution range including distinct habitats, allowing the comparison between populations exposed to different environmental and human density parameters.

Although selection of a broader pathogen testing panel and a wide range of hosts would ensure the description of a more complex and realistic epidemiological scenario of the interaction between pathogens and threatened Iberian leuciscids, selection of mesophilic *Aeromonas* spp. as a pathogen model and of *Iberochondrostoma lusitanicum* as the host model allowed a more detailed investigation of the primary goals of this thesis.

Hence, specific goals were pursued with each of the experimental works developed throughout this thesis, namely:

1) To determine the prevalence, diversity, virulence and antimicrobial resistance signatures of mesophilic *Aeromonas* spp. in distinct *I. lusitanicum* populations, as well as health parameters such as body condition and extension of skin lesions in this species, and to identify differences in these parameters regarding origin of the individuals and sampling season;

2) To compare mesophilic *Aeromonas* spp. diversity and skin lesion extension in two sympatric leuciscid species – *I. lusitanicum* and *Squalius pyrenaicus* – and to explore differences in bacterial populations between the two fish species;
3) To evaluate the role of biosafety measures applied in the Portuguese *ex situ* breeding program for endemic leuciscids, specifically for *I. lusitanicum*, on the prevalence, diversity, antimicrobial resistance and virulence signatures of mesophilic *Aeromonas* spp.;
4) To test the influence of water temperature and pH predicted climacteric alterations as projected by the Intergovernmental Panel on Climate Change (IPCC) on the antimicrobial resistance signatures and biofilm production of single and mixed cultures of mesophilic *Aeromonas* spp.

Chapter 2. Molecular epidemiology, virulence traits and antimicrobial resistance signatures of *Aeromonas* spp. in the critically endangered *Iberochondrostoma lusitanicum* follow geographical and seasonal patterns

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Conceptualization, MLG, CSS, JIR and MO; field work, MLG and CSS; laboratory work, MLG and SI; data analysis, MLG and LC; statistical analysis, CSM and TAM; writing—original draft preparation, MLG; writing—review and editing, MLG, SI, LC, CSM, TAM, CSS, JIR and MO; supervision, JIR and MO, project administration, MO; funding acquisition, MLG, JIR and MO. All authors have read and agreed to the published version of the manuscript.

2.1. Abstract

Despite the fact that freshwater fish populations are experiencing severe declines worldwide, our knowledge on the interaction between endangered populations and pathogenic agents remains scarce. In this study, we investigated the prevalence and structure of *Aeromonas* communities isolated from the critically endangered *Iberochondrostoma lusitanicum*, a model species for threatened Iberian leuciscids, as well as health parameters in this species. Additionally, we evaluated the virulence profiles, antimicrobial resistance signatures and genomic relationships of the *Aeromonas* isolates. Lesion prevalence, extension and body condition were deeply affected by location and seasonality, with poorer performances in the dry season. *Aeromonas* composition shifted among seasons and was also different across river streams. The pathogenic potential of the isolates significantly increased during the dry season. Additionally, isolates displaying clinically relevant antimicrobial resistance phenotypes (carbapenem and fluroquinolone resistance) were detected. As it inhabits intermittent rivers, often reduced to disconnected pools during the summer, the dry season is a critical period for *I. lusitanicum*, with lower general health status and a higher potential of infection by *Aeromonas* spp. Habitat quality seems a determining factor on the sustainable development of this fish species. Also, these individuals act as reservoirs of important antimicrobial resistant bacteria with potential implications for public health.

2.2. Introduction

Past geological events that shaped freshwater landscapes in the Iberian Peninsula and promoted its geographical isolation greatly contributed to the high level of endemism currently observed in native leuciscid fishes (Teleostei: Leuciscidae) (Sousa-Santos et al. 2019). In spite of this biotic richness, leuciscids' populations are following the decreasing trends observed in freshwater species worldwide (Reid et al. 2019), and around 70% of the known species in the region belonging to this taxonomic group present some level of threat to their conservation (Doadrio et al. 2011). Several attempts are currently implemented to counter current trends, including habitat restoration and *ex situ* breeding programs targeting several species (Sousa-Santos et al. 2014b).

Different factors play a key role as potential threats for the sustainability of leuciscid species. Namely, habitat degradation and destruction due to anthropogenic actions such as the construction of dams and weirs, water abstraction (e.g., for agriculture), river architecture alteration, increased pollution levels due to direct or land run-offs, and the introduction and proliferation of exotic species are known to impact these species' survival (Maceda-Veiga 2013). For species living in the Mediterranean area, climate fluctuations are another key issue

and are suggested to become more important in the future. Leuciscids that occur in intermittent streams of the southern part of the Iberian Peninsula are typically subjected to cyclical shifts between winter floods and extremely low water levels during the summer. In the dry season, the incidence of droughts increases in these streams, fragmenting the river and restricting movement and habitat availability for fishes (Sousa-Santos et al. 2016). This situation is likely to get worse with predicted climatic changes (Jarić et al. 2019).

The loss of river connectivity seasonally imposed by the lowering water levels in the region often results in a series of more or less disconnected summer pools. It is common to observe a high number of individuals gathered in these small habitats where, due to a higher depth, water remains available—although with no flow, higher temperatures and lower dissolved oxygen levels (Magoulick and Kobza 2003). Higher fish densities likely contribute to the deterioration of water quality parameters and to an increase of intra and inter-species competition for resources and individual contact; which potentially influences host's immune function, with obvious consequences for overall fitness (Sousa-Santos et al. 2016; Marcos-López et al. 2010; Whitney et al. 2016). Despite these conditions being ideal for the spread of several pathogenic agents, the prevalence and impact infectious diseases might have in these populations is not clear so far. Investigations regarding the prevalence of specific pathogens are limited and often do not focus on the impact these agents might have for the host. Also, past studies have focused on parasitological and fungal surveys (Sánchez-Hernández 2017; Aller-Gancedo et al. 2016), neglecting the influence that other pathogenic agents—i.e., bacteria and virus—might have in leuciscids.

Bacterial pathogens are common infectious agents in fish species and assume particular relevance in this context (Austin and Austin 2016). When the water temperature increases, which is associated with the higher fish densities observed in the summer pools, microbial loads also tend to increase (North et al. 2014). Members of the genus *Aeromonas* can be important in this situation, since infections caused by these bacteria are considered one of the most common type of bacterial infection detected in freshwater fish species, causing morbidity and mortality in both farmed and wild fishes (Austin and Austin 2007). Indeed, they are commonly known pathogenic agents ubiquitous in several aquatic ecosystems and are also accounted as both zoonotic agents and antibiotic resistance and virulence reservoirs (Janda and Abbott 2010; Baron et al. 2017; Cai et al. 2019; Zhou et al. 2019).

It is important to understand the host-pathogen dynamics of these agents and how they can impact these already imperilled populations and constitute significant threats to their conservation. Identifying specific pathogens that might contribute to the decrease of a fish species' stock and understanding how these pathogen communities are shaped by environmental conditions is the first step in creating species and habitat recovery plans that

take into account specific susceptibilities for a population in particular and help to implement strategies to restore habitats, fighting detrimental impacts pathogenic agents might have.

Developing active sampling schemes, using subsets of individuals, can help to investigate both the prevalence and impact of selected pathogens—e.g., *Aeromonas* spp.—and produce valuable information for the interested stakeholders. Moreover, patterns among different populations from the same species occupying diverse geographical areas or between different seasons can potentially be discriminated during these sampling actions and result in the definition of risk factors which would contribute to a better management of the species. Additionally, and since there is a growing concern about the role that aquatic ecosystems play as end-point collectors of determinants of antibiotic resistance and virulence originating from land (Chen et al. 2018), using such sampling schemes as opportunistic actions to survey and detect antimicrobial resistance and virulence in natural aquatic habitats is of major importance to signal and prevent dissemination points between the interface land-water (Grilo et al. 2020). The microbiota of wild species is of particular significance since wildlife plays an active role in the dissemination of microbiota and genetic determinants in natural environments (Hu et al 2017). Also, *Aeromonas* spp. are known to express a great variety of virulence factors involved in host colonization and invasion, constituting a possible virulence reservoir in the aquatic environment (Harnisz and Korzeniewska 2018). Additionally, the potential of *Aeromonas* spp. to acquire and disseminate resistance determinants is widely acknowledged, making them relevant indicators of antimicrobial resistance in aquatic environments (Piotrowska and Popowska 2015).

We hypothesize that *Aeromonas* communities associated with distinct populations of endangered leuciscids will vary according to the geographical origin of the populations— i.e., different exposure to environmental and anthropogenic conditions—and season (dry and wet). In addition, we suggest that distinct patterns of fish fitness (e.g., body condition and skin conformation) are present in populations of the same species. Also, we hypothesize that antimicrobial resistance signatures and virulence traits of the *Aeromonas* isolates vary according to different factors (i.e., location and season). To test these ideas, we used isolated wild populations of the critically endangered boga portuguesa (*Iberochondrostoma lusitanicum*; Collares-Pereira 1980), a model species for endangered leuciscids, evaluated their skin lesion scores, body condition and *Aeromonas* spp. prevalence, composition, similarity relationships, virulence profiles and antimicrobial resistance signatures of the isolates.

2.3. Materials and Methods

2.3.1. Sampling site description and fish sampling

The selection of sampling sites followed a set of criteria to fulfil study inclusion. Namely: 1) the current known distribution of *I. lusitanicum*, established based on annual monitoring census performed by our team (Sousa-Santos et al. 2016); 2) the level of water abstraction in the potential sampling sites, accessed two weeks prior to sampling by on-site observations, to estimate fish presence; 3) different levels of water quality and environmental parameters between basins (pH, temperature, dissolved oxygen, total dissolved solids, electrical conductivity, nitrites, nitrates, phosphates), as determined from previous monitoring census (Sousa-Santos, personal communication); 4) different levels of mean human population density in the civil communities closely associated with the sampling sites, as determined by the Census 2011 Program (INE 2011). In total, four river basins were selected (Lizandro, Samarra, Jamor, Laje), representing different examples of rural and urban environments with distinct water quality conditions. Sampling was performed in previously characterized sites (Lizandro: 38.886701˚, −9.298140˚; Samarra: 38.894761˚, −9.433734˚; Jamor: 38.720832˚, −9.249696˚; Laje; 38.709159˚, −9.314079˚; Figure 8) (Sousa-Santos et al. 2016). Additionally, to compare differences among bacterial prevalence and skin alteration scores in the dry and in the wet season, two sampling actions were conducted in October 2017 and February 2018, respectively.

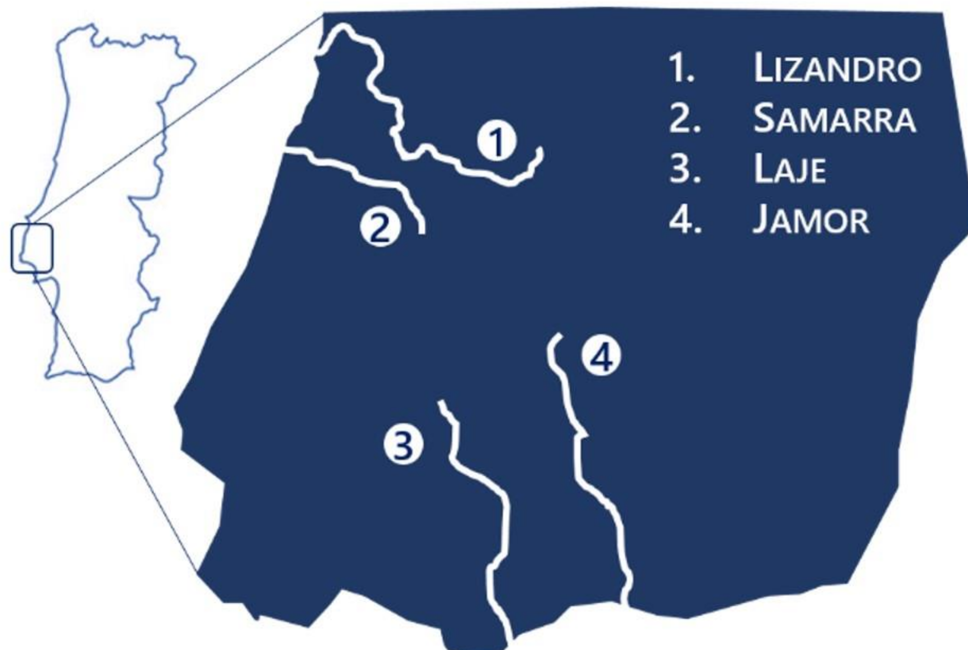

Figure 8. Study area.

I. lusitanicum was used as a model species to study *Aeromonas* prevalence in wild endangered leuciscid species. *I. lusitanicum* is a leuciscid fish, endemic to Portugal, only occurring in small coastal streams of the West Region and in the larger Tagus and Sado river basins. Its selection was based on the following criteria: 1) presenting a relevant conservation threat status (critically endangered), as defined by the IUCN (Crivelli 2021), representing the potential of bacterial infections to disrupt imperilled leuciscid populations; and 2) the occurrence in distinct stream types throughout the species' distribution area, aiming to evaluate the influence of ecological and anthropogenic factors on *Aeromonas* epidemiology and antimicrobial resistance and virulence profiles.

The sampling protocol followed was similar at each sampling site, involving electrofishing by two operators. Fishing period was timed (from the onset of electrofishing until the collection of the last individual). One operator surveyed the stream in parallel transects in the downstream–upstream direction and collected *I. lusitanicum* individuals following standard electrofishing procedures (CEN 2003). As the animals were collected, the second operator transferred them to a container with water collected at site to allow monitoring of the stunning recuperation (consequence of the electrofishing process) and guarantee fish survival.

After recuperation, all collected animals were individually inspected for the presence of lesions commonly associated with *Aeromonas* spp. infection (i.e., haemorrhagic lesions and skin ulcers). The prevalence of lesions was recorded for each individual. A set of sixteen individuals displaying lesions was randomly selected in every sampling location for further analysis and lesion location and description was recorded. Each individual was transferred to a measuring device to allow measuring of fork length (measured from the tip of the snout to the notch of the caudal fin) and taking pictures from both sides (when possible) to record body condition and lesions extent. In each set, two age groups with 8 individuals were created based on fork length (juveniles <40 mm; adults ≥40 mm). Sex was also determined by visual inspection, which was possible for some adults during the wet season (prior to the breeding season, when sexual dimorphism is more evident: females exhibit swollen abdomens and males show nuptial tubercules at the top of their heads). Finally, water excess was wiped from each individual's body using a sterile gauze and a bacteriological sample was collected from skin areas with typical lesions using an ESwab™ LiquidAmies Collection and Transport System (ThermoFisher Scientific®, Waltham, MA, USA). After this step, the full recuperation of the animal was confirmed (normal swimming behaviour and respiratory rate), following by its release into the river stream. Swabs were stored at 4 °C until further processing. Bacterial isolation was performed at the Laboratory of Microbiology and Immunology of the Faculty of Veterinary Medicine, University of Lisbon, Portugal.

Habitat conditions at each sampling site were characterized regarding connectivity (i.e., fishes isolated in summer pools vs. fishes present in stretch with hydrological connectivity) and physical and chemical parameters: 1) pH, temperature, total dissolved solids and electrical conductivity; using a portable waterproof pH meter model HI98130 (Hanna Instruments®, Woonsocket RI, USA); 2) dissolved oxygen, using a waterproof oxygen meter model 9146-10, with probe HI76407/10F (Hanna Instruments®); and 3) nitrites, nitrates and phosphates, using colorimetric strips (ITS Thorsten BetzelTM, Hattersheim, Germany).

2.3.2. Body condition score (BCS)

Photographs of lateral views from each individual were used to access the BCS using a visual score ranging from 1 to 5 produced for adult zebrafish (Clark et al. 2018), with minor adaptations. The analysis was based on two anatomical references, as suggested by Clark et al. (2018), and included: 1) the relationship between the width of the cranial region between the eye and the operculum and the abdominal girth at a halfway position between the pectoral fin and the dorsal fin; and 2) the curvature of the ventral surface of the body. While fat deposition in the ventral surface of the zebrafish occurs more cranially (Clark et al. 2018), in *I. lusitanicum* the deposition is homogeneous and the ventral surface does not create a protrusion. When available, a score was given for each lateral view and an average score from both sides was produced; otherwise, scores resulted from single lateral views. Female individuals sampled in the wet season were excluded from the analysis since BCS was likely altered by egg development.

2.3.3. Skin lesion score (SLS)

Photographs of each individual were used to produce an individual skin lesion score by determining the level of lesions in the skin macroscopic morphology (namely, the presence of ulcerations and areas with haemorrhage). Each photograph was analysed by computer image software (ImageJ, NIH, Bethesda, MD, USA) and the skin lesion score was produced as such: (total area of skin presenting lesions/total body area) ×100. Fins, except for the caudal fin, were excluded from the body area under analysis since it was not possible to observe them in all animals. A score was obtained from the analysis of each lateral view of every individual (when possible) and a final average score was obtained per animal.

2.3.4. *Aeromonas* isolation

Swabs collected from each individual were inoculated in tubes with 8 mL of Brain Heart Infusion (BHI) broth (VWR, Radnor, PA, USA), vortexed, and incubated at 37 °C for 24 h. Samples were transferred to Glutamate Starch Red Phenol (GSP) agar plates supplemented with 100,000 IU sodium penicillin g/L (Merck, Kenilworth, NJ, USA), a selective and differential agar medium for *Aeromonas* spp., and incubated at 37 °C for 12 h. Large (2–3 mm) yellow colonies surrounded by a yellow zone were presumptively identified as *Aeromonas* spp. and four distinct colonies were randomly selected for each individual and isolated into pure cultures in BHI Agar (37 °C for 24 h). Isolates were characterized regarding Gram-staining and oxidase activity and stored in Buffered Peptone Water (VWR) with 20% glycerol at −80 °C during the study. *Aeromonas hydrophila* ATCC 7966 was used as a positive control.

2.3.5. Genomic typing

Bacterial genomic DNA was obtained by the boiling method as described in Talon et al. (1998). Genomic typing of the isolates was conducted using a Random Amplified Polymorphic DNA (RAPD) technique. The method was used as described before (Szczuka and Kaznowski 2004; Barroco 2013), with minor modifications. Primers AP3 and AP5 (Szczuka and Kaznowski 2004) were used in independent mixtures to perform isolates' typing (STABVIDA, Caparica, Portugal). Each amplification reaction was performed in a final volume of 25 µL. The mixture consisted of: 12.5 µL of Supreme NZYTaq 2× Green Master Mix (NZYTech, Lisbon, Portugal), 8.5 µL of PCR-grade water (Sigma-Aldrich, St. Louis, MO, USA), 2.5 µL of Bovine Serum Albumine (0.01%; Thermo Fisher Scientific), 0.5 µL (1 µM) of primer and 1 µL of template DNA. Thermocycler conditions were as follow: 94 °C for 5 min; 40 cycles of 94 °C for 45 s, 40 °C for 1 min, and 72 °C for 2 min; and 72°C for 5 min.

Amplification products were resolved using agarose gel electrophoresis with 1.5% (w/v) agarose in 1× TBE Buffer (NZYTech). Gels were resolved for 50 min at 90 V. NZYDNA Ladder VII (NZYTech) was used as a molecular weight marker. The visualization of gels was performed using a UV light transilluminator and images were recorded trough the Bio-Rad ChemiDoc XRS imaging system (Bio-Rad Laboratories, Hercules, CA, USA).

2.3.6. *Aeromonas* species identification

A multiplex PCR protocol, previously described by Persson et al. (2015), discriminating between *A. caviae*, *A. media*, *A. hydrophila* and *A. veronii* based on *gyrB* and *rpoB* genes was used, with minor modifications. *A. caviae* ATCC 1976, *A. hydrophila* ATCC 7966, *A. media* ATCC 33907 and *A. veronii* ATCC 35624 were used as positive controls.

PCR mixtures were performed in a final volume of 25 µL and were composed of: 12.15 µL of Supreme NZYTaq 2× Green Master Mix (NZYTech), 10 µL of PCR-grade water (Sigma-Aldrich), 0.25 µL (0.5 µM) of primers A-cav, 0.225 µL (0.45 µM) of primers A-hyd, 0.1 µL (0.2 µM) of primers A-med, 0.075 µL (0.15 µM) of primers A-ver, 0.025 µL (0.05 µM) of primers A-16S; and 1.5 µL of template DNA. Thermocycler (VWR) parameters included hot start at 95°C for 2 min; followed by six cycles of denaturation at 94 °C for 40 s, annealing at 68°C for 50 s, and extension at 72 °C for 40 s; and 30 cycles at 94 °C for 40 s, 66 °C for 50 s, and 72 °C for 40 s.

PCR products were resolved by agarose gel electrophoresis as described before. Gels were resolved for 45 min at 90 V. NZYDNA Ladder VI (NZYTech) was used as a molecular weight marker. Gels were visualized and recorded as described before.

2.3.7. Virulence factors' evaluation

Virulence factor expression was evaluated using phenotypical assays according to protocols previously described, with minor modifications. Specifically, haemolytic activity was accessed using Columbia agar supplemented with 5% sheep (VWR) for 24 h (Santos et al. 1999); lipase activity was determined using Spirit Blue Agar (Difco, Franklin Lakes, NJ, USA) supplemented with 0.2% Tween 80 (VWR) and 20% olive oil (commercial) for 8 h (Blaise and Armstrong, 1973); gelatinase activity was evaluated using Oxoid™ Nutrient Gelatin (Thermo Fisher Scientific) for 24 h (Han et al. 2008); protease activity was detected using Skim Milk Agar (Sigma-Aldrich) for 24 h (Mellergaard 1983); DNase activity was established using DNase Test Agar with Methyl Green (VWR) for 24 h (Hickey et al. 2013); and the production of slime was determined using Congo Red Agar for 72 h (Freeman et al. 1989). Incubation temperature was established based on river's water temperature data collected during annual monitoring census performed in the summer seasons of 2017 to 2019 (Sousa-Santos, personal communication) and averaged (22 °C). This was performed in order to mimic fish's body temperature since they are poikilothermic.

Each isolate's virulence index was calculated based on the ratio between positive tests for virulence factors and the total amount of virulence factors evaluated (Singh et al. 2017).

The following strains were used as controls: *A. caviae* ATCC 15468 (haemolysin negative), *A. hydrophila* ATCC 7966 (DNase and haemolysin positive), *Enterococcus faecium* EZ40 clinical isolate canine periodontal disease (slime producer), *Escherichia coli* ATCC 25922 (DNase and gelatinase negative; slime non-producer), *Pseudomonas aeruginosa* Z25.1 clinical isolate diabetic foot infection (protease and gelatinase positive; lipase negative) and *Staphylococcus aureus* ATCC 29213 (lipase positive, protease negative). *P. aeruginosa*

and *E. faecium* (Mendes et al. 2012, Semedo-Lemsaddek et al. 2016) belong to the bacterial collection of the Laboratory of Microbiology and Immunology, Faculty of Veterinary Medicine, University of Lisbon, Portugal.

2.3.8. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was determined using the disk diffusion technique (Bauer et al 1966), as established in the guidelines and following breakpoints of the Clinical and Laboratory Standards Institute (NCCLS 2002; CLSI 2013, 2015). *E. coli* ATCC 25922 was used as a quality control. The following antibiotics (Mastdiscs®, Mast Group, Liverpool, UK) were evaluated: amikacin (AK, 30 µg), amoxicillin/clavulanic acid (AUG, 20–10 µg), aztreonam (ATM, 30 µg), ceftazidime (CAZ, 30 µg), enrofloxacin (ENF, 5 µg), erythromycin (E, 15 µg), florfenicol (FFC, 30 µg), imipenem (IMI, 10 µg), nitrofurantoin (NI, 300 µg), streptomycin (S, 10 µg), tetracycline (T, 30 µg) and sulfamethoxazole/trimethoprim (TS, 23.75–1.25 µg). Antimicrobial compound choice followed those commonly used to treat Gram-negative infections in Human and Veterinary Medicine, with a special focus in aquaculture.

Isolates were categorized as multidrug-resistant when presenting non-susceptibility (intermediate and resistant status) to at least one antimicrobial compound in three or more antimicrobial categories (Magiorakos et al. 2012). Multiple Antibiotic Resistance (MAR) index values were produced for each isolate and calculated as described by Krumperman (1983). Antimicrobial compounds to which *Aeromonas* spp. are considered intrinsically resistant (amoxicillin/clavulanic, erythromycin and streptomycin) were not included in the multidrug resistance characterization and in the MAR index calculation.

2.3.9. Data and statistical analysis

To analyse the reproducibility level of the molecular species identification, phenotypic virulence expression, antimicrobial susceptibility testing and genomic typing, a random sample including 10% replicates was used.

BioNumerics® version 7.6 software (Applied Maths, Sint-Martens-Latem, Belgium) was used to evaluate genomic typing relationships. Fingerprints similarity was found based on a dendrogram calculated with the Pearson correlation coefficient. An optimization value of 0.5% was used. Cluster analysis was achieved through the unweighted pair group method with arithmetic average (UPGMA). The reproducibility value was determined as the average similarity value of all replicate's pairs (87.6%) with patterns presenting higher similarity values considered to be undistinguishable. Regarding isolates considered clones, one was randomly

selected and only distinct strains were considered for further analysis (species identification, virulence factor's screening, antimicrobial susceptibility testing and statistical analysis).

The diversity of the typing profiles between locations in each season was evaluated using the Simpson index (D) (Simpson 1949). Clones were excluded from the analysis and clusters of molecular types were formed based on an integrated evaluation of the fingerprinting profiles and the different *Aeromonas* species. The lowest similarity value by cluster was investigated and the same value was applied to each dendrogram to enable further comparisons.

Pearson's correlations were calculated between: 1) fish size and the BCS, 2) size and the SLS, 3) the SLS and the BCS, at the fish level, and 4) the MAR index and the virulence index, at the isolate level.

Several response variables evaluated at each site and sampling season were modelled as a function of season and origin. A binomial logistic GLM was considered for the proportion of individuals with skin lesions. A beta response (continuous values ranging from 0 to 1) was considered for the Simpson index.

Additionally, some fish level variables were modelled as a function of season and origin. A linear model was considered for both (1) BCS and (2) SLS.

Several isolate level response variables were modelled as a function of season, origin and *Aeromonas* species. Using a binomial logistic GLM, the (1) multidrug resistance of an isolate (0—No, 1—Yes) and (2) the virulence factors (0—Negative, 1—Positive) were considered. Using a beta response (continuous values ranging from 0 to 1) the (3) MAR and (4) virulence indexes were considered. Also, several isolate level response variables were modelled as a function of season and origin. Using a binomial logistic GLM, antimicrobial susceptibility was considered. Using a multinomial log-linear model (package nnet, version 7.3-15) (Venables and Ripley 2002), the proportion of the different species of *Aeromonas* was considered. To accommodate the possible non-independence in isolate level responses across fish, a GLMM framework with fish as a random effect was implemented but the results indicated that there was a residual amount of variability associated with that random effect and changes to results would be negligible. Hence, reporting of the results was based on the simpler GLM analysis.

Regarding the influence of cluster on SLS and BCS, a gaussian GLM was applied. Since many of the bacterial clusters had a low number of observations, all clusters with less than four observations were pooled and generated as a new cluster serving as an intercept for the model. Additionally, and since the same individual fish could display strains in more than one bacterial cluster, only one isolate observation was randomly chosen for each

individual. To incorporate the variability that is induced by the loss of information of using a single isolate at each iteration, this process was repeated a thousand times by sampling random isolates at each iteration. Histograms of the p-values obtained for each of these 1000 iterations were produced and it was assumed that significant results would lead to histograms where a large proportion of the corresponding p-values would be significant. The statistical analysis was done using R software (R Core Team 2021).

2.4. Results

The fishing efforts in this study allowed for sampling 406 *I. lusitanicum* individuals in both seasons and all four locations (dry season—285; wet season—121). Furthermore, formation of groups based on age stratification (adults and juveniles) was achieved in both sampling campaigns.

Lesion prevalence in sampled individuals varied according to season, being significantly lower in the wet season ($p<0.001$). In the individuals collected during sampling actions, there was a significantly higher proportion of fishes displaying lesions ($p<0.001$) when compared to those who did not display any lesions. Among sampled locations, when compared to the Laje location, significantly lower levels of lesion prevalence were observed in individuals originating from Lizandro ($p=0.002$) and significantly higher levels of lesion prevalence in individuals originating from Samarra ($p<0.001$).

The sampled rivers presented differences regarding flow regime. Water physical and chemical parameters, as well as site characterization, are presented in Table 3.

Body condition scores (BCS) differed significantly regarding location ($p<0.001$). Individuals originating from Laje presented higher BCS, followed by those from Lizandro, Jamor and Samarra. No association between BCS and sampling season was found ($p=0.104$), although a trend was observed with higher scores in the wet season. There was a positive correlation between BCS and the size of the animal ($r=0.46$; $p<0.001$).

Skin lesion score (SLS) of the individuals was significantly different between seasons and locations ($p<0.001$). SLS was significantly lower in the wet season. Individuals from Samarra river presented higher SLS values than individuals from other locations. There was a negative correlation between individual’s size and SLS ($r=-0.28$; $p<0.001$) and a negative correlation between SLS and BCS ($r=-0.17$; $p=0.015$).

Table 3. River's water physical and chemical parameters and characterization regarding connectivity and human density level. C—continuous stream, I—isolated pool, R—rural, U—urban.

	Dry Season				Wet Season			
	Lizandro	Samarra	Laje	Jamor	Lizandro	Samarra	Laje	Jamor
pH	7.95	8.07	8.11	8.07	8.08	8.25	9.62	9.09
Temperature (°C)	19.4	19.9	19.9	19.2	10.9	12.9	14.2	14.9
Dissolved Oxygen (ppm)	9.58	13.41	11.74	10.39	12.9	13.69	13.63	13.59
Total Dissolved Solids (ppm)	0.41	0.57	0.53	0.33	0.44	0.49	0.43	0.3
Electrical Conductivity (mS)	0.8	1.15	1.05	0.66	0.87	0.97	0.43	0.3
Nitrites (mg/L)	0	0	0.15	0.15	0.15	0.5	0.15	0
Nitrates (mg/L)	0	0	0	0	0.5	2	0.5	0
River connectivity	C	I	I	C	C	C	C	C
Density level	R	R	U	U	R	R	U	U

Aeromonas spp. prevalence was high in individuals from both seasons and locations (dry season=100%, wet season=92.2%). In total, a bacterial library of 376 isolates of *Aeromonas* was established. After clone elimination, 223 isolates were further studied. Species composition differed among locations and seasons ($p<0.001$), revealing specific structures for each river in each season (Figure 9). In general, *A. hydrophila* was the most prevalent species isolated from the sampled animals in both seasons, despite reaching higher prevalence values in those from the dry season, followed by *A. veronii* and *A. media*. In the individuals sampled in the dry season, a predominance of *A. hydrophila* was observed in the Jamor river, outnumbering other species, while fishes from Samarra river presented higher levels of *A. veronii*. In the wet season, despite high levels of *A. hydrophila* in the sampled animals, individuals sampled in Jamor presented higher levels of *A. caviae*. On the other hand, animals from Laje presented higher values of *A. media* and *A. veronii*. The prevalence of *A. veronii* in fishes from Samarra was lower than the prevalence registered in the dry season.

Virulence indexes were significantly influenced by sampling season and the species of the isolates ($p<0.001$). Isolates collected in the animals from the wet season scored significantly lower than those collected in animals from the dry season. Regarding the species of *Aeromonas*, *A. caviae* isolates presented the higher virulence indexes, followed by *Aeromonas* spp., *A. hydrophila*, *A. veronii* and *A. media*. No correlation between the virulence index and the multiple antibiotic resistance (MAR) index was found ($r=-0.12$, $p=0.081$).

All the tested isolates (n=222) displayed lipolytic activity. A total of 204 isolates (92%) had hemolytic activity, 89% of the isolates produced DNase activity, 86% exhibited proteolytic activity and 40% of the isolates were slime producers. Only 24% of the isolates exhibited gelatinase activity.

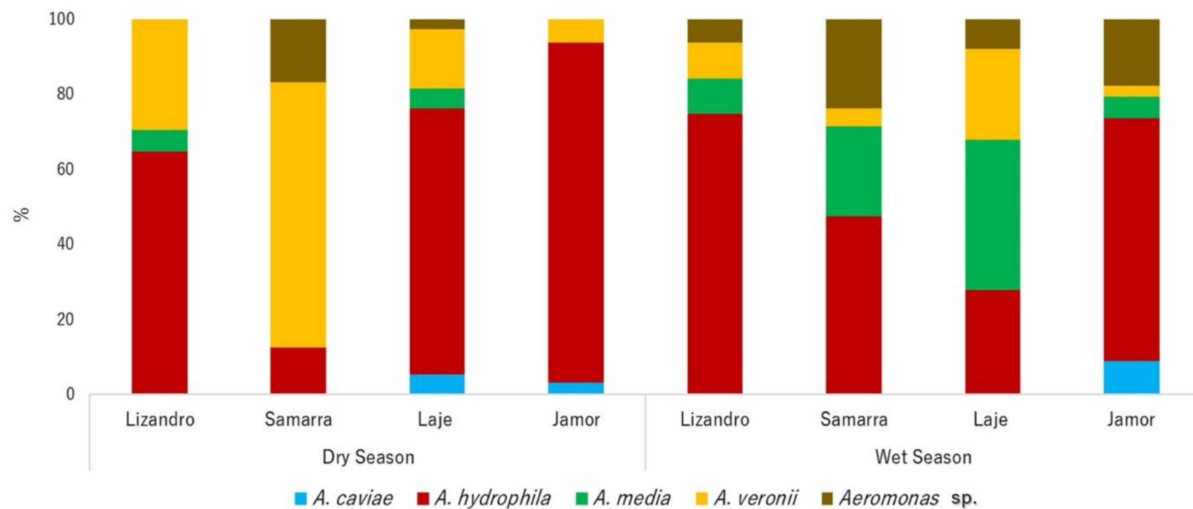

Figure 9. Relative abundance of *Aeromonas* species by location and sampling season.

Regarding the virulence signatures registered for isolates from each sampling location, shifts occurred for selected virulence factors between isolates from different locations and across seasons (Figure 10). Slime production varied significantly between isolates from different locations ($p<0.001$), with higher prevalence in those from samples collected in Samarra, while the majority of the slime-producing strains were *A. veronii*. DNase prevalence was lower in isolates collected in the wet season ($p<0.001$) and influenced by the origin ($p<0.001$), with lower positive prevalence in those sampled in Laje river. Most of the DNase-negative isolates obtained in samples from this river were *A. media*. Regarding gelatinase prevalence, a higher positive prevalence was recorded on isolates from the dry season and from Lizandro and Jamor ($p<0.001$). The vast majority of these isolates were *A. hydrophila*. For hemolytic activity, only season affected the prevalence of positive results ($p=0.012$), with isolates from samples collected in the dry season presenting more positive results. Regarding protease, the sampling season influenced the prevalence of positive results ($p=0.024$), since the isolates from dry season samples presented a higher prevalence of this virulence factor.

Among the studied bacterial collection, 30.2% (n=67) of the isolates were considered multidrug resistant and none showed resistance to all the antimicrobials tested. The prevalence of multidrug resistant was significantly influenced by sampling season ($p<0.001$),

with a higher prevalence of such isolates being obtained from samples collected in the wet season (73.1%, n=49). Similarly, the MAR index of the isolates also differed significantly among seasons (p<0.001), with isolates collected from samples during the wet season presenting significantly higher scores. Isolates collected from samples from Jamor river presented the higher values, followed by those collected in Laje, Samarra and Lizandro. The MAR index values for the different *Aeromonas* species did not differ significantly (p=0.837).

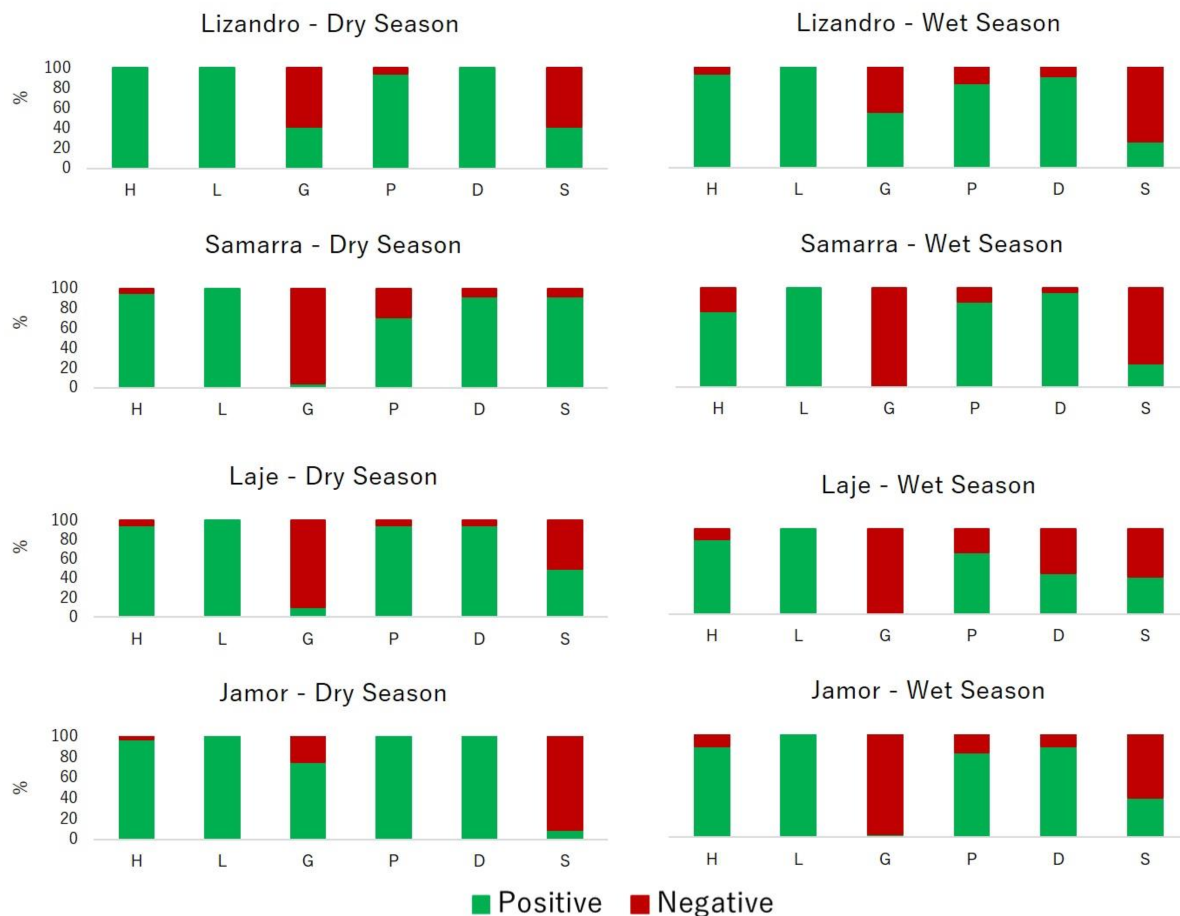

Figure 10. Relative prevalence of virulence factors by location and sampling season. H—haemolytic activity, L—lipase activity, G—gelatinase activity, P—protease activity, D—DNase activity, S—slime production.

Higher levels of non-susceptibility (intermediate and resistant categories) were recorded for erythromycin (100%), amoxicillin/clavulanic acid (98%) and streptomycin (61%). Moderate levels were registered for enrofloxacin (40%), sulfamethoxazole/trimethoprim (34%), imipenem (34%), amikacin (22%) and ceftazidime (19%). The lowest non-susceptibility

levels were recorded for tetracycline (11%), florfenicol (8%), nitrofurantoin (8%) and aztreonam (4%).

Regarding the isolates' resistance profile for each antimicrobial tested, fluctuations in the relative proportion of each susceptibility category occurred for the evaluated panel of antibiotics, both between locations and seasons (Figure 11). Regarding the results for ceftazidime, there was a higher prevalence of isolates presenting a non-susceptible status in samples from the wet season ($p<0.001$) when compared to the dry season and a lower prevalence of non-susceptible status in those from Lizandro ($p=0.027$) when compared to Jamor. In relation to enrofloxacin, similar trends to ceftazidime regarding season ($p<0.001$) and location ($p=0.023$) were observed. Regarding florfenicol ($p=0.006$), nitrofurantoin ($p=0.015$), tetracycline ($p=0.002$) and sulfamethoxazole/trimethoprim ($p<0.001$), a higher prevalence of isolates with a non-susceptible status were registered in the wet season. For streptomycin, a lower prevalence of isolates with non-susceptible status occurred in Laje ($p=0.011$) when compared to Jamor.

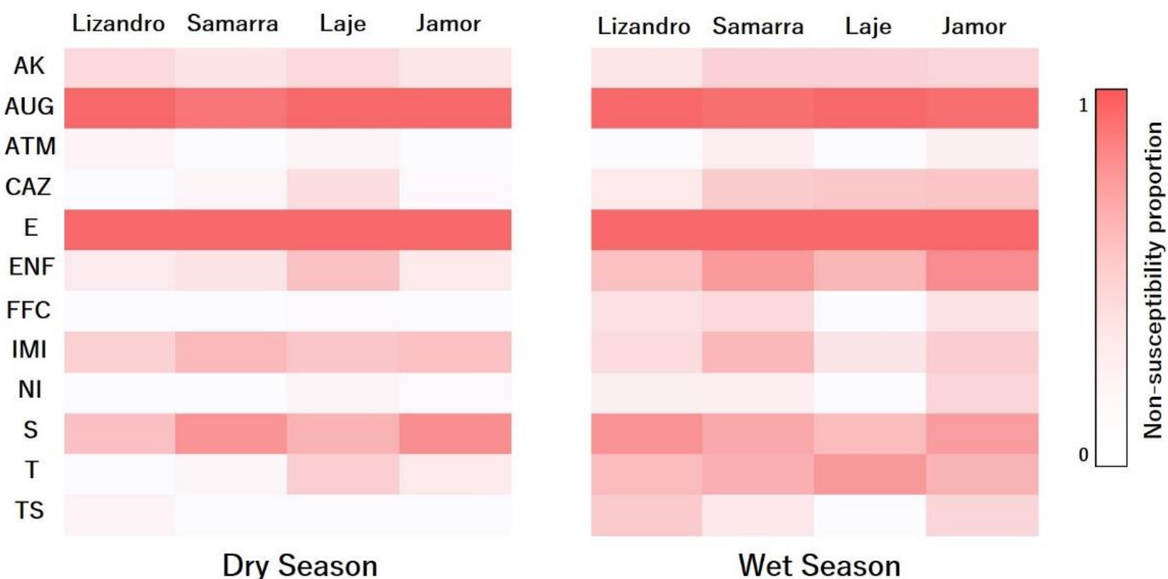

Figure 11. Relative prevalence of non-susceptibility to the tested antimicrobial compounds of the isolates by location and sampling season. AK—amikacin, AUG—amoxicillin/clavulanic acid, ATM—aztreonam, CAZ—ceftazidime, E—erythromycin, ENF—enrofloxacin, FFC—florfenicol, IMI—imipenem, NI—nitrofurantoin, S—streptomycin, T—tetracycline, TS—sulfamethoxazole/trimethoprim.

Molecular typing of the isolates and their relations evaluated by dendrogram analysis revealed a high number of clusters at the 66.98% of similarity (based on a joint evaluation of

fingerprinting patterns, *Aeromonas* species, river of origin of the isolate and season in which was collected) among the studied collection (Supplementary Figure S1, Annex II). Several clones were identified at the 87.6% similarity (reproducibility level), corresponding to isolates originating from the same animal or from different animals in the same river. However, no persistence of clones was observed for samples obtained in the same location across seasons nor the presence of clones originating from samples from different rivers.

Regarding each season, specific trends were observed (Figure 12). In the dry season, four clusters were formed (based on the fingerprinting patterns, *Aeromonas* species and river of origin of the isolates). Cluster I, defined at 14.36% similarity, has 47 isolates and is mainly composed by isolates of *A. veronii*, with the presence of *A. hydrophila* and *A. media* isolates. The majority of isolates in this cluster was obtained from individuals from Samarra river. Cluster II, defined at 23.12% similarity, has 68 isolates and is mainly composed by *A. hydrophila* isolates, although *A. caviae*, *A. media* and *Aeromonas* sp. were also present. Origin of the isolates was more disperse, although a big contribution from samples from animals collected in Laje river is noted. Cluster III, defined at 32.91% similarity, has 13 isolates and is exclusively formed by *A. hydrophila* from animals from Lizandro river, and is mainly composed (84.6%) by clones retrieved from four distinct *I. lusitanicum*'s individuals. Cluster IV, defined at 38.43%, has 32 isolates and is mainly composed of *A. hydrophila* isolates, with a small proportion of *A. caviae* and *A. veronii*. The vast majority of the isolates were collected from animals in Jamor river, where clones of *A. hydrophila* (11 isolates) were recovered from six individuals.

In the wet season, eight clusters were determined (following the same parameters used for dry season). Cluster I, defined at 25.26% similarity, has 20 isolates and is mainly composed of *A. veronii* isolates, with additional *A. hydrophila*, *A. media* and *Aeromonas* sp. isolates. The majority were obtained from animals collected in Laje river. Cluster II, defined at 41.18% similarity, has 39 isolates and is mainly constituted by *A. hydrophila* isolates, with few *A. caviae* isolates. The vast majority of the isolates originated from samples collected in Jamor river. Cluster III, defined at 42.8% similarity, has 44 isolates and groups mainly *A. hydrophila* isolates with *A. media*, *A. veronii* and *Aeromonas* sp. also included. The isolates were obtained from fishes of the four rivers almost proportionally. Cluster IV, defined at 44.08% similarity, has eight isolates, all identified as *A. hydrophila* from samples collected in Lizandro river. Cluster V, defined at 26.31% similarity, has six isolates and is mainly composed of *A. veronii* and one isolate of *A. media*. The majority of the isolates were from samples collected in Laje river. Cluster VI, defined at 24.3% similarity, has 40 isolates and a predominance of *A. media* and *A. hydrophila*, but also includes *A. veronii* and *Aeromonas* sp. Samples collected in Laje and Samarra rivers contributed to a higher proportion of the isolates. Cluster VII, defined at

44.54% similarity, has four isolates, including *A. veronii*, *A. hydrophila* and *A. media*, from samples collected in Lizandro, Jamor and Samarra rivers. Cluster VIII, defined at 50.28% similarity, has six isolates, half of them corresponding to *A. media*, but also including *Aeromonas* sp. and *A. hydrophila*. All of the isolates from animals sampled in the Laje river.

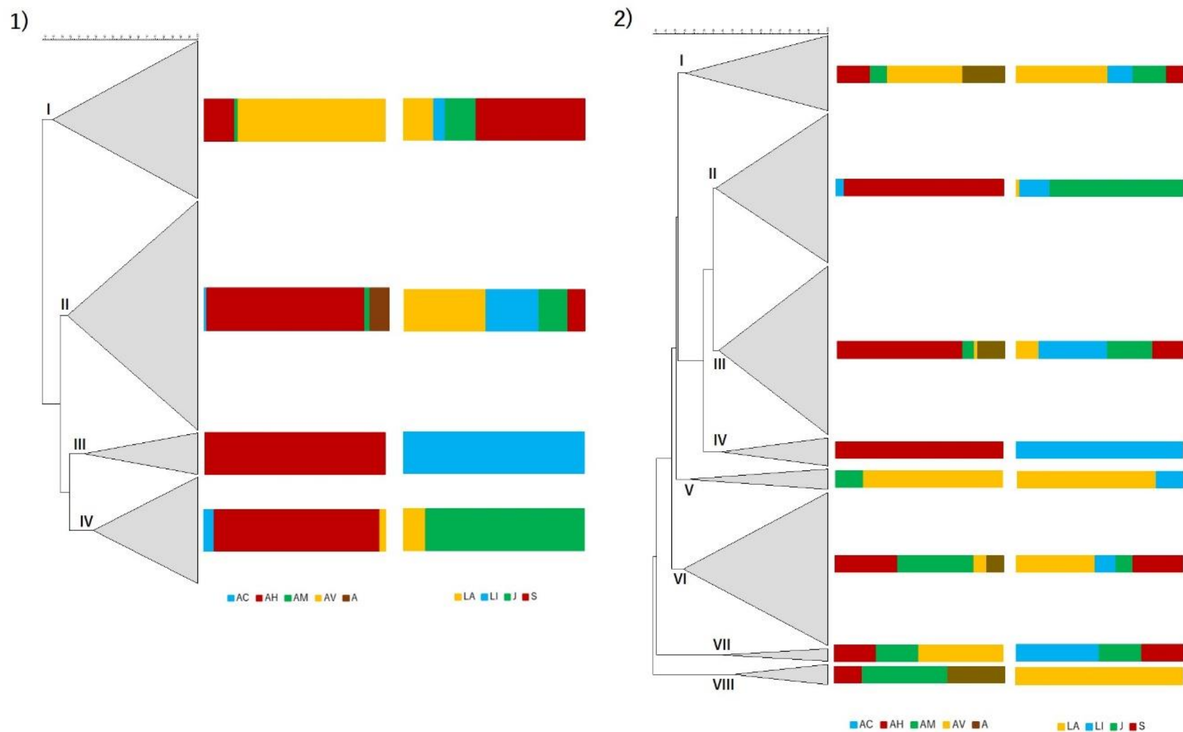

Figure 12. Hierarchical numerical analysis of the isolates recovered from samples collected in the dry season (1) and wet season (2). The dendrograms were based in the PCR fingerprints obtained with the AP5 and AP3 primers in a composite approach using for similarity calculation the Pearson correlation coefficient. Clustering was achieved with UPGMA. Regarding the entire dendrograms, cophenetic correlation coefficients are 0.83 for the dry season and 0.79 for the wet season. The scale used represents the percentage of similarity between the PCR fingerprints. The first column represents the dendrogram, the second the relative abundance (%) of Aeromonas species and the third the relative proportion (%) of isolates based on the river of origin. AC—*A. caviae*, AH—*A. hydrophila*, AM—*A. media*, AV—*A. veronii*, A—*Aeromonas* sp., LA—Laje, LI—Lizandro, J—Jamor, S—Samarra.

When investigating isolates' genomic relationships in each location, it is possible to form clusters that reflect the sampling season (Figure 13). That is particularly obvious for the Jamor river, where most of the isolates grouped in two clusters, each with a majority of isolates for one same sampling season. While such separation was also present in the other sampling locations, some clusters presented isolates belonging to both seasons.

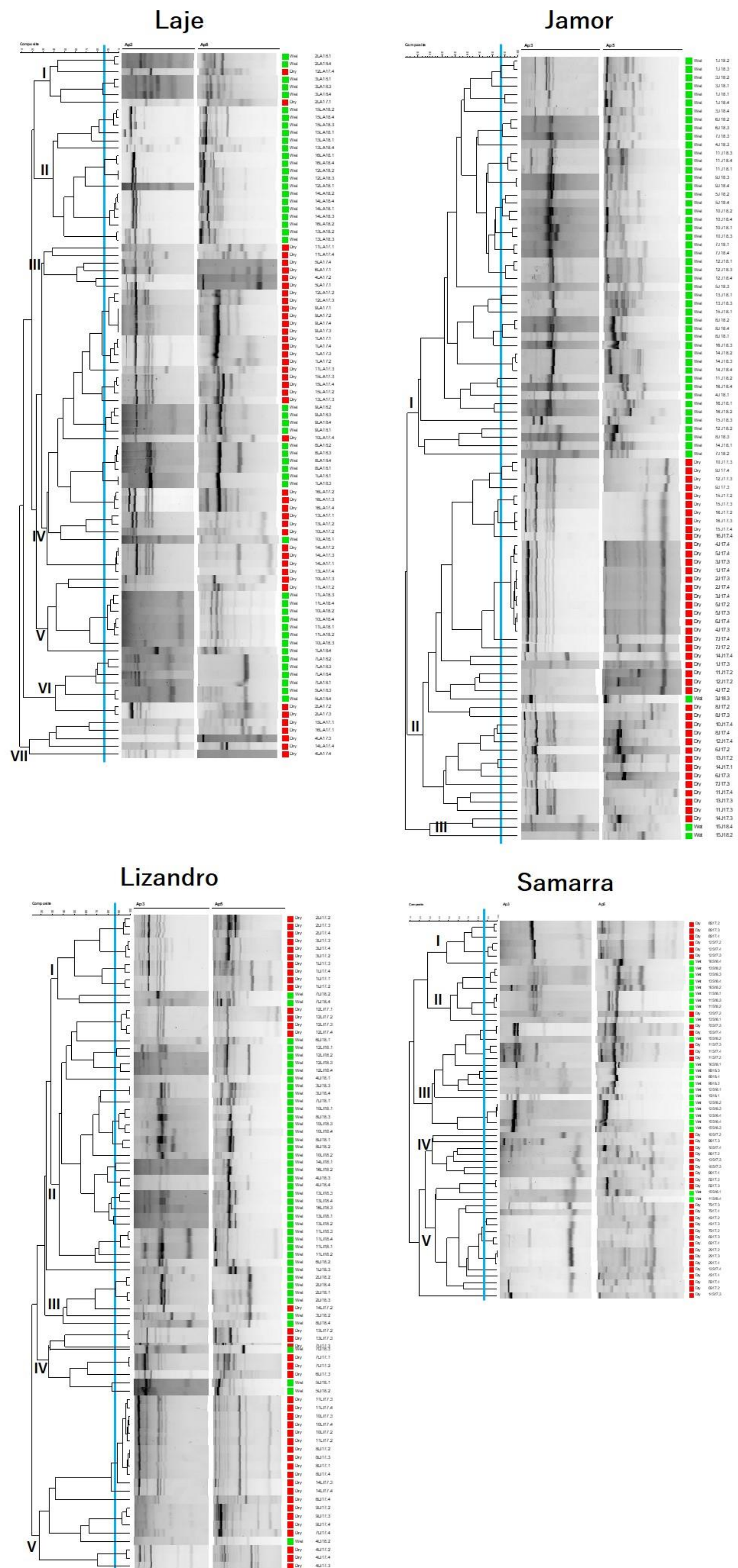

Figure 13. Dendrograms of isolates based on the composite analysis of RAPD fingerprints with primers Ap3 and Ap5 (Pearson correlation coefficient and UPGMA clustering) from each of the sampling locations. Cophenetic correlation coefficients are as follows: Lizandro—0.85, Samarra—0.86, Laje—0.83, Jamor—0.87. Differential coloration represents sampling season in which the isolate was collected: Red—Dry Season; Green—Wet Season.

Regarding the genomic relationships of the identified *Aeromonas* species, some trends were observed (Supplementary Figures S2–S5, Annexes III-VI). Isolates of *A. caviae*, *A. hydrophila* and *A. veronii* presented clusters composed of a majority of isolates collected during a specific sampling season. This differentiation was particularly obvious in the case of *A. caviae*. Such differentiation was not present in the dendrogram of *A. media* isolates, although a low prevalence of this species in the dry season was recorded. Such grouping was not visible regarding the origin of the isolates, although some clusters were in their vast majority represented by isolates from samples collected in the same river (e.g., *A. hydrophila* in Jamor and Lizandro, *A. veronii* in Samarra, Laje and Lizandro). Based on the cluster evaluation (Supplementary Figures S6 and S7, Annexes VII and VIII), Simpson's diversity index did not differ across seasons (p=0.448). Diversity indexes were considered high in both seasons (DDry season=0.97; DWet season=0.96). The indexes differed between locations, namely the diversity indexes recorded in Laje were significantly higher than those in the Jamor river (p=0.011; Supplementary Figures S8–S15, Annexes IX-XVI).

Regarding the analysis of the association between bacterial clusters and fish characteristics, the presence of a non-conventional data structure hinders conclusive results (namely, the fact that several isolates were obtained from the same fish, which induces a dependence across isolates, and hence different clusters within fish). There is absence of evidence that any given cluster lead to higher or lower values of SLS or BCS.

2.5. Discussion

Surveillance programs have the potential to unravel biotic and abiotic drivers of pathogen presence, composition and host-pathogen interaction. Such schemes can produce valuable information often lacking in natural habitat management plans. Although skin lesions in fishes can have a multifactorial origin, with several possible different agents involved and contribution of *Aeromonas* spp. to the lesions observed in *I. lusitanicum* cannot be concluded, the methodology applied in this study can access dynamics of bacterial pathogens with relevance for conservation medicine and, at the same time, serve as an opportunistic basis for the study of antimicrobial resistance and virulence prevalence and temporal shifts with a particular relevance for public health. In this study, we show that distinct populations of the critically endangered leuciscid fish *I. lusitanicum* display differential levels of general health with shifts according to seasons and locations, and that those modifications are accompanied by changes in the *Aeromonas* structure associated with them. Furthermore, the structure, antimicrobial resistance profile and virulence signatures

of these *Aeromonas* isolates are shaped by seasonal and spatial drivers. Finally, we consider that *I. lusitanicum* populations are acting as reservoirs of isolates with clinically important antibiotic resistance phenotypes.

2.5.1. Body condition score (BCS) and skin lesion score (SLS)

A higher prevalence of lesions and higher scores of SLS were detected in the dry season, and most of the individuals sampled during this season displayed skin lesions. This high incidence of cases reinforces the idea that the dry season, when several threats to freshwater fish have a simultaneous and cumulative negative impact (Maceda-Veiga 2013), constitutes a temporal frame of fragility for the fitness and consequent survival of this endangered species. Fish skin responds quickly to changes in the environment (Noga 2000), and several stressors are known to trigger the cellular damage that results in skin lesions such as those observed in this study (Law 2001). Namely, oxygen depletion, exposure to xenobiotic chemicals and biotoxins, pH and temperature fluctuations, high levels of organic matter, parasites, stress and confinement are known to be correlated with the onset of skin diseases, both in wild and cultured fishes (Plumb et al. 1976; Ventura and Grizzle 1987; Noga 2000; Udomkusonsri and Noga, 2005; Murawski et al. 2014; Granneman et al. 2017; Lamb et al. 2018; Vajargah et al. 2018). Additionally, and once skin damage is established and the homeostatic mechanism of the host is affected, optimal grounds for bacterial colonization and invasion are established, favouring the proliferation of opportunistic pathogens such as *Aeromonas* spp. (Ventura and Grizzle 1987). This phenomenon was already observed in combination with a variety of stressors (Plumb et al. 1976; Austin 1998; Marques et al. 2016) and, in the case of motile aeromonads, likely benefits from warmer temperatures that potentiate bacterial proliferation (Cipriano and Austin 2011).

Like skin lesions, fishes' body condition also varies according to the stressors present in their environment. Growth and body condition have been reported to be negatively correlated with reduced water quality, pollution levels in the environment, lower prey abundance and habitat degradation (Suns and Hitchin 1990; Maceda-Veiga et al. 2014; Granneman et al. 2017; Lamb et al. 2018; Cavraro et al. 2019). Interestingly, although *I. lusitanicum* suggested prey items, such as macroinvertebrates and zooplankton, are available in the dry season (Chiu et al. 2018; Pinto 2018), their diversity and abundance decrease in streams highly impacted by pollution and hydric stress (Kalogianni et al. 2017; Arenas-Sánchez et al. 2021). This situation likely generates intra-specific and interspecific competition for food, particularly with invasive species that are better adapted to environmental critical conditions (Marques 2016; Vedia et al. 2019). It is also likely that, as

observed for other species, *I. lusitanicum* individuals would change their dietary focus into plant material and detritus under critical conditions (Alexandre et al. 2015). This negative dietary balance experienced by fish individuals will affect their ability for disease resistance, since a balanced nutritional intake is key for the immune system competence (Jiang et al. 2017).

Both SLS and BCS were correlated with individual size: while smaller individuals displayed in average the highest SLS, they were also the ones scoring the lowest regarding their BCS. Such findings highlight the vulnerability of younger life stages towards the adverse conditions (i.e., overcrowding, decrease levels of food availability, predation) they encounter in their natural habitat, coupled with an immature immune system that does not allow them to fully prevent disease by pathogenic agents (Cornet et al. 2020). For this reason, bacterial infections in early life stages of fish are common (Wang et al. 2008). Also, SLS levels were negatively correlated with BCS. Although causality direction is difficult to establish in this case, this finding suggests that homeostasis disruption in *I. lusitanicum* individuals is accompanied by overall deleterious effects that can compromise fish survival.

A marked differentiation in SLS and BCS occurred between animals sampled in different locations. In the Samarra population, a consistent higher prevalence of lesions and SLS was detected in the sampled individuals, and the BCS was also the lowest in average from the animals in all sampled locations. Contrarily, in fishes from Lizandro, the prevalence of lesions was the lowest. River characteristics, both regarding water quality and anthropogenic influences, seem to shape the individual homeostatic state and characterize the onset of dysregulations that culminate in the physical expression of lesions. Although, in general, all the studied streams present medium to low water quality indexes (BDJUR 2002; Antunes 2021; Lopes 2014), the Samarra River presented the higher levels of total dissolved solids and electrical conductivity, suggestive of higher content of organic matter. In fact, in its last kilometres, this river is influenced by effluents from a wastewater treatment plant (Lopes 2014). Bad water quality, restricted habitats (reduced summer pools) and habitat degradation are possible synergetic drivers that influence the overall health of *I. lusitanicum* and likely result in the observed changes for the Samarra population. In the Lizandro River, despite being influenced by agricultural runoffs (Oliveira et al. 2011), it seems that the sampled location provides a habitat compatible with the sustainable development of a *I. lusitanicum* population. These findings highlight the role that habitat quality have in the prevalence of healthy *I. lusitanicum* populations.

2.5.2. *Aeromonas* prevalence, structure, similarity relationships and diversity

Regarding *Aeromonas* prevalence, a high level of isolates was detected in animals sampled in both seasons and across all locations. *Aeromonas* spp. prevalence in the aquatic habitat seems to be highly dependent on the climacteric conditions associated with the water (Pathak et al. 1988). Several studies report a predominance of *Aeromonas* prevalence during winter, with a significant decrease of bacterial levels during the summertime (Pathak et al. 1988; Rhodes and Kator 1994). This is likely the case in regions where a strong temperature fluctuation is noted between seasons, while in temperate regions the opposite occurs and the climacteric conditions favour the establishment of bacterial populations all-year round, with a predominance when water temperature rises during summer months (Seidler et al. 1980; Cavari et al. 1981; Pettibone 1998; Maalej et al. 2003; Chaix et al. 2017). However, it has been proposed that *Aeromonas* prevalence in fishes remains stable throughout the year (Pathak et al. 1988), as observed in our study. This is particularly important since fishes, besides being affected by pathogenic aeromonads, can also constitute reservoirs and maintain *Aeromonas* communities during adverse climacteric conditions. Even in the case that a determined bacterial strain fails to induce disease, an individual can constitute an important disseminator for other conspecifics that might be experiencing decreased or immature immunological functions (e.g., juveniles) and establish outbreaks in a population. This possibility is supported by our findings that demonstrate the sharing of bacterial clones among several individuals in each location, which is likely a consequence of the gregarious behaviour of *I. lusitanicum*.

In our study, the predominant *Aeromonas* species detected in *I. lusitanicum* were *A. hydrophila*, *A. veronii* and *A. media*. *A. caviae* was detected in a lower proportion. These findings are in agreement with previous results of prevalence studies conducted with fishes (Radu et al. 2003; Perretta et al. 2018; Popović et al. 2019; Rather et al. 2019). However, some studies report differences in *Aeromonas* prevalence in fish species (Beaz-Hidalgo et al. 2010; Popovic et al. 2015; Ran et al. 2018; Wu et al. 2019; Borella et al. 2020). This is probably a reflection of differential environmental pools of *Aeromonas* communities to which these fish species are exposed, coupled with host genotype adaptations that lead to particular associations of host and microbiota. The role of *A. veronii* as a primary pathogen involved in motile aeromonad septicaemia has been emphasized, which has been attributed to the role aerolysin has in the virulence of this species (Ran et al. 2018). In our study, a predominance of *A. veronii* was recorded for individuals belonging to the Samarra river's population during the dry season. The highest prevalence of skin lesions and the highest scores of SLS were also recorded in this population during the dry season, establishing a possible link with *A. veronii* prevalence. It is noteworthy that *A. veronii* may

be linked with primary cases of infection in these individuals, contradicting the idea that *A. hydrophila* is the most virulent species for aquatic animals. *A. caviae* has previously been appointed as dominant in waters contaminated with sewage and is considered an indicator of faecal contamination (Araujo et al. 1991; Popovic et al. 2015). Interestingly, in our study this species was only isolated in individuals from Jamor and Laje streams, the two locations associated with urban settings. It seems that the recovery of *A. caviae* from these locations exposes these streams as more impacted by sewage disposal, a likely consequence of urbanization (Araujo et al. 1991).

Our results differ from other studies focusing on the prevalence and diversity of *Aeromonas* species in freshwater streams (Chowdhury et al. 1990; Araujo et al. 1991; Khor et al. 2015; Popovic et al. 2015; Chaix et al. 2017; Solaiman e Micallef 2021). However, and although the aquatic environmental compartment is the most likely route for the acquisition of these isolates, the *Aeromonas* communities colonizing *I. lusitanicum* individuals probably differs in proportion regarding their environmental counter partners. Chaix et al. (2017) showed that the *Aeromonas* communities associated with copepods differed regarding the communities available in the water column. This phenomenon mirrors bacterial adaptations regarding host colonization, signalling the success determined strains have in adhering to and colonizing animal hosts when compared with others present in the environmental pool.

The *Aeromonas* communities retrieved from *I. lusitanicum* individuals varied across sampled locations and seasons, revealing specific profiles for each combination of variables. Water temperature has been proposed as a limiting factor shaping *Aeromonas* prevalence (Popovic et al. 2015), and the results retrieved from the molecular typing in this study seem to corroborate this association. Similarity relationships among the isolates were highly shaped by the sampling season, as described before (Popovic et al. 2015), suggesting that biotic characteristics differing among dry and wet season determine the bacterial community structure in each sampling time. Additionally, no strain permanence was observed in any location between seasons. Also, no clones were detected in different locations and the bacterial clustering was influenced by location, supporting the idea that each stream presents autochthonous populations specific to the conditions established in a determined location. In our study, however, it is not possible to determine if this fluctuation in the bacterial diversity is a sole product of temperature, since water physical and chemical parameters varied in all locations across seasons. Other factors have been implied in *Aeromonas* prevalence in aquatic streams, such as the redox potential, conductivity, organic matter level, pH, dissolved oxygen, total dissolved nitrogen, phosphate levels and water turbidity (Hazen 1979; Pathak et al. 1988; Araujo et al. 1991; Rhodes and Kator 1994; Pianetti et al. 2006). It is likely that the

observed *Aeromonas* communities in *I. lusitanicum* are a result of the input of multifactorial biotic and abiotic aspects, shaping the final community structure.

A high bacterial diversity was observed in both seasons. The diversity levels differed between some locations (i.e., Laje and Jamor), revealing the influence of the presence of determined clones and hence the predominance of some strains in a given location. Although these clones were not widely prevalent across our study, they expose the success certain strains have to colonize a higher number of individuals, consequently being more prevalent. Additionally, it is possible that the conditions experienced in both locations drive the establishment of more successful bacterial clusters in relationship to adverse factors present in the streams. Colin et al. (2021) exposed how urbanization of freshwater streams favours the abundance of determined bacterial groups (i.e., *Aeromonas* spp.) and leads to decreasing levels of bacterial diversity in fishes' skin. In our study, Jamor population is the one with higher human population density in its vicinity, and the observed reduced diversity levels are a probable consequence of the stressors fish are exposed to, namely the degree of water pollution (Araujo et al. 1991; Colin et al. 2021).

2.5.3. Virulence factors

The majority of the virulence factors studied, as well as the virulence indexes, were more prevalent and higher in isolates from animals sampled in the dry season. Several factors have been proposed to modulate the regulation of bacterial virulence gene expression. Water temperature has an important role in virulence gene expression, induced by bacterial temperature sensor systems that detect temperature shifts and trigger changes in gene expression (Guijarro et al. 2015; Rasmussen-Ivey et al. 2016a). In mesophilic aeromonads, increases in temperature are associated with higher virulence and mortality, associated with the upregulation of specific virulence pathways and the increased production of extracellular toxins (Guijarro et al. 2015; Pattanayak et al. 2020). Nutrient availability, and specifically of nitrogenous compounds (Karunakaran e Devi 1995), influences the activation of metabolic pathways. When water presents higher organic matter, bacteria possess available energy to increase their virulence gene expression and intensify infectivity (Nagar et al. 2016; Zhang et al. 2020). Contrarily, in situations of nutrient deprivation, virulence gene expression is downregulated as a measure to save energy (Casabianca et al. 2015). Additionally, the exposure to stress hormones from the host (i.e., norepinephrine) increases the expression of a wide array of virulence genes (Gao et al. 2019). It can be concluded that the environmental conditions of the dry season (i.e., warmer waters, higher nutrient load and

increased fish stress due to overpopulation) result in an amplified virulence expression by *Aeromonas* spp., as demonstrated by our results.

The virulence indexes varied across the *Aeromonas* species. Globally, *A. caviae* and *Aeromonas* sp. presented the highest indexes. This finding is probably related to a sampling bias, since these groups were substantially smaller than the other sampled species. Several pan-genomic analyses performed in the genus *Aeromonas* report a hierarchization in virulence potential among the several species, related to the abundance and diversity of virulence genes (Ghatak et al. 2016; Zhong et al. 2019; Talagrand-Reboul et al. 2020). Among them, *A. hydrophila* is generally considered one of the most pathogenic species, followed by *A. veronii*. *A. media*, on the other hand, is considered to display a low virulence profile (Talagrand-Reboul et al. 2020).

Virulence in the *Aeromonas* genus results from a multifactorial array of virulence factors, including extracellular products and slime production (Fernández-Bravo and Figueras, 2020). High prevalence of lipolytic, haemolytic, DNase and proteolytic activity observed in this study are in accordance with results from previous investigations (Radu et al. 2003; Carvalho et al. 2012; Miyagi et al. 2016; Chenia et al. 2017; Wu et al. 2019; Das et al. 2020). Similarly, low prevalence of gelatinolytic activity has also been observed before (Sechi et al. 2002), as well as equivalent levels of slime production (Hossain et al. 2020; Wickramanayake et al. 2020). However, prevalence values and trends seem to vary among surveillance studies (Yano et al. 2015; Chenia et al. 2017; Igbinosa et al. 2017; Dias et al. 2018; Hossain et al. 2020; Muduli et al. 2021), suggesting that virulence signatures of a specific *Aeromonas* community are the result of an interaction between the community's composition and the environmental factors shaping their habitat. This is possibly why specific associations of variations in prevalence of extracellular products and slime production and selected streams were observed in this study.

2.5.4. Antimicrobial resistance

Although some variability can be observed, members of the genus *Aeromonas* are described as generally being resistant to penicillins, narrow spectrum cephalosporins, macrolides (clarithromycin) and antifolates (sulfamethoxazole), while presenting susceptibility to aminoglycosides, carbapenems, fluoroquinolones, extended-spectrum cephalosporins, monobactams, nitrofurans, phenicols and tetracyclines (Janda and Abbott 2010; Harnisz and Korzeniewska 2018; Fernández-Bravo and Figueras, 2020). Our results corroborate these general definitions and are in accordance with previous surveys (Radu et al 2003;

Scarano et al 2018; Dahanayake et al. 2019; Popović et al. 2019; Wu et al. 2019; Borella et al. 2020), although some variations exist for specific compounds that likely reflect local dynamics characteristic to each study area. *Aeromonas* intrinsic resistance to many β-lactam antibiotics is widely acknowledged and it is the result of the combination of the constitutive expression of a several array of β-lactamases with an external membrane with a low permeability (Bello-López et al. 2019). In our study, it is noteworthy that almost a third of the isolates presented non-susceptibility to carbapenems, an antimicrobial class considered of last resource. Tacão et al. (2015) reported a high incidence of *bla*CphA genes, conferring resistance to carbapenems, in *Aeromonas* spp. collected from the Vouga river basin (Portugal). The role of wild animals (i.e., nutria) in serving as reservoirs of carbapenem-resistant *Aeromonas* was previously showed (Lim et al. 2019). Similarly, quinolone resistance is also recognized in the genus *Aeromonas* (Varela et al. 2016). In our study, 40% of the isolates presented non-susceptibility to enrofloxacin. Our results show the establishment of antibiotic resistance phenotypes in bacteria from small riverine ecosystems in the Lisbon district. Furthermore, we stress the role of wild species, like *I. lusitanicum*, as reservoirs of clinically important resistance determinants in these environments.

In total, 30% of the isolates detected in this study were considered multidrug resistant. While the prevalence of multidrug resistant *Aeromonas* varies among studies, several authors report high levels of multidrug resistance in their surveys among this genus (Piotrowska and Popowska 2015; Dias et al. 2018; Perretta et al. 2018; Borella et al. 2020). In a recent investigation with *Aeromonas* isolates collected from water samples in the river Tua (northern Portugal), Gomes et al. (2021) determined that 83.3% of the studied collection was multidrug resistant. Although it is alarming that about a third of the isolates in this study present a multidrug resistance phenotype, current results suggest that sampled locations might be under lower selective pressure than other streams in the national territory and abroad. The observed variations in the resistance signatures of the isolates collected in each location and season likely reflect the demographic dynamics to which these streams are exposed and are a mirror of the resistome characteristic to the anthropogenic communities (both humans, domestic animals and crops) in close association with them.

In this study, both the MAR index scores and the prevalence of multidrug resistant strains were higher in the wet season. Some studies report an increase in antibiotic residues, antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) in the dry season (Guo et al. 2018; Liang et al. 2020). This can be linked as a direct consequence of the decrease in water levels observed in warmer months, resulting in a concentration effect in the streams. However, building evidence expose the wet season as a period when resistance

determinants, especially ARGs, increase (Knapp et al. 2012; Li et al. 2018; Harnisz et al. 2020). Rainfall is considered a major driver of ARGs prevalence in river streams, since rainfall events increase the transfer of resistance determinants from terrestrial settings (e.g., urban, agricultural) to water bodies through runoffs (Di Cesare et al. 2017; Huang et al. 2019; Stange and Tiehm 2020). Contrary to chemical pollutants, ARGs impact is less likely to be hampered by the dilution effect caused by the rainfall (Peng et al 2020). In our study, the sampling performed during the wet season was preceded by the most intense raining events of the year (November–January) which possibly translated into the differences in antibiotic resistance between seasons observed. Similarly, such runoff events can also explain the differences in MAR index scores observed across isolates from different sampling locations. Higher scores were recorded in isolates from animals sampled in the Jamor and Laje rivers, two streams located in urban settings and associated with higher human population densities. Human-impacted environments play an important role in increasing and promoting the transmission of ARGs in rivers, and the prevalence of ARGs increases with urbanization (Pruden et al. 2012). Specific anthropogenic activities, such as agricultural runoffs, urban wastewater discharging and antimicrobial therapy use, promote a selective pressure in the microbiota present in that environment, shaping its antibiotic resistance signatures (Peng et al. 2020). It seems that the resistome of a river is highly influenced by its level of urbanization, revealing the risk that urban rivers pose as reservoirs of resistance in aquatic environments.

2.6. Conclusions

Current results expose differences in fish fitness across four populations of *I. lusitanicum* from the Lisbon district (Portugal), suggesting that geography and habitat quality play a key role in the sustainable development of the species. Additionally, our findings stress the vulnerability *I. lusitanicum* individuals face seasonally, being the dry season a critical period for this species. This study also produces further knowledge regarding the epidemiology of *Aeromonas* spp. in this species, shedding light on their interaction with *I. lusitanicum* and how they shift seasonally and spatially. Finally, we confirmed the presence of clinically relevant antimicrobial resistance phenotypes in *Aeromonas* isolated from *I. lusitanicum*, confirming both their potential as resistance indicators in aquatic environments and the role of *I. lusitanicum* individuals as reservoirs of zoonotic antibiotic resistant bacteria in river streams of the Lisbon district.

Studying the general health of Iberian leuciscids and their interactions with potentially pathogenic *Aeromonas* spp. deepens our knowledge on the ecology of these species and the threats they experience. This is especially relevant for two reasons. First, it complies with

international legislation, such as the Water Framework Directive in Europe, in its requirement to investigate methodology to evaluate the ecological status of aquatic environments using sentinel species, such as the ones used in this study. Second, it provides important knowledge regarding the signalization of leuciscids' populations facing higher risk of extinction that can be used in a more rational species management program.

Several knowledge gaps still exist regarding the interaction between *Aeromonas* spp. and Iberian leuciscids. Further research should focus on the evaluation of susceptibility patterns to this bacterial genus by the different species of endangered Iberian leuciscids, to pinpoint the species most at risk. Similarly, a wider evaluation of the impact other possible health stressors (e.g., infectious, pollutants, stress) have in these species and how could their control be implemented in management programs should be conducted. Finally, microbial source tracking techniques should be used to determine the origin of antimicrobial resistance determinants reported in this study to prevent introduction in wild aquatic habitats and consequent establishment of reservoirs.

Chapter 3. Sympatric threatened Iberian leuciscids exhibit differences in *Aeromonas* diversity and skin lesions' prevalence

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Conceptualization, MLG, TAM, CSS, JIR and MO; data curation, MLG and CSS; formal analysis, MLG, LC, TAM and CSS; funding acquisition, MLG, JIR and MO; investigation, MLG and CSS; methodology, MLG, LC, TAM, CSS and MO; project administration, JIR and MO; resources, MLG, CSS and MO; software, MLG, LC and TAM; supervision, JIR and MO; validation, MLG, LC, TAM, CSS, JIR and MO; visualization, MLG, CSS, JIR and MO; writing – original draft, MLG; writing – review & editing, MLG, LC, TAM, CSS, JIR and MO.

3.1. Abstract

Assessments regarding health aspects of Iberian leuciscids are limited. There is currently an information gap regarding effects of infectious diseases on these populations and their role as a possible conservation threat. Moreover, differences in susceptibility to particular agents, such as *Aeromonas* spp., by different species/populations is not clear. To understand potential differences in *Aeromonas* diversity and load, as well as in the prevalence and proportion of skin lesions, in fishes exposed to similar environmental conditions, an observational study was implemented. Using a set of 12 individuals belonging to two sympatric Iberian leuciscid species (*Squalius pyrenaicus* and *Iberochondrostoma lusitanicum*), the skin lesion score in each individual was analyzed. Furthermore, a bacterial collection of *Aeromonas* spp. isolated from each individual was created and isolates' load was quantified by plate counting, identified at species level using a multiplex-PCR assay and virulence profiles established using classical phenotypic methods. The similarity relationships of the isolates were evaluated using a RAPD analysis. The skin lesion score was significantly higher in *S. pyrenaicus*, while the *Aeromonas* spp. load did not differ between species. When analyzing *Aeromonas* species diversity between fishes, different patterns were observed. A predominance of *A. hydrophila* was detected in *S. pyrenaicus* individuals, while *I. lusitanicum* individuals displayed a more diverse structure. Similarly, the virulence index of isolates from *S. pyrenaicus* was higher, mostly due to the isolated *Aeromonas* species. Genomic typing clustered the isolates mainly by fish species and skin lesion score. Specific *Aeromonas* clusters were associated with higher virulence indexes. Current results suggest potential differences in susceptibility to *Aeromonas* spp. at the fish species/individual level, and constitute important knowledge for proper wildlife management through the signalization of at-risk fish populations and hierarchization of conservation measures.

3.2. Introduction

Freshwater habitats are among the most threatened ecosystems worldwide and this is reflected in the conservation status of their biodiversity (Darwall and Freyhof 2015). Freshwater populations have declined at an alarming rate in the last 40 years (Reid et al. 2019), while freshwater fishes have the largest extinction rate among vertebrates in the 21st century (Baumsteiger and Moyle 2017). Specific life traits, such as small body size, shorter longevity and small distribution range, predispose these species for extinction (Kopf et al. 2017; Liu et al. 2017; Jarić et al. 2019), although geographical origin is also accounted as a preponderant factor. Species endemic to areas such as the Mediterranean region are particularly at risk, and this is expected to be aggravated by predicted climacteric alterations (Jarić et al. 2019).

In the Iberian Peninsula, a high level of leuciscid species' endemism is observed (Sousa-Santos et al. 2019). This phenomenon resulted from past geological events that shaped freshwater ecosystems and promoted the isolation of ancestral evolutionary lineages, previously inhabiting interconnected paleobasins, in confined regions and/or river courses (Filipe et al. 2009). Despite the high level of speciation in leuciscids in the Iberian freshwater networks, around 70% of these species are listed under a threatened conservation status (Doadrio et al 2011). These species occur mainly in small Mediterranean-type river basins. These are typically influenced by a high seasonality, with the incidence of floods in the winter and droughts during the summer (Sousa-Santos et al. 2016). The droughts' period can result in the fragmentation of the river into disconnected pools, which congregates individuals, decreases habitat quality and jeopardizes fish survival. Additional recognized threats for these species include water abstraction, damming, specific agricultural practices, water pollution, and introduction and proliferation of invasive and exotic species (Maceda-Veiga 2013).

Health assessments in Iberian leuciscids are scarce (Perez-Bote 2000). Despite conservational efforts developed in recent years to mitigate the impact different threats have in these endangered species, investigations focusing on health parameters are needed. In particular, the role of infectious diseases in modulating freshwater fish populations is poorly known (Gutiérrez-Galindo and Lacasa-Millán 2005; Sánchez-Hernández 2017). Understanding how infectious diseases in general, or relevant pathogens for each population, impact populational fitness and compromise the species' sustainable development is fundamental. The acquisition of such knowledge can contribute to the establishment of habitat and species recovery plans that account for populational specific susceptibilities and implement strategies to restore habitats into normal equilibriums.

In this context, bacterial pathogens – such as the members of the genus *Aeromonas* – are of particular relevance. *Aeromonas* spp. are widely acknowledged for their pathogenic potential in aquatic animals, especially in fishes. Several reports implicate species of *Aeromonas* as the morbidity and/or mortality cause of wild and cultured fishes (Austin and Austin 2007). This bacterial genus is globally dispersed in several aquatic environments (Janda and Abbott 2010) and has increasingly gained importance as a zoonotic agent and antimicrobial resistance indicator, especially regarding the emergence of multidrug resistance (Baron et al. 2017; Grilo et al. 2020). Virulence in the *Aeromonas* genus is associated with a wide range of virulence factors, such as the production of slime or extracellular products (Fernández-Bravo and Figueras 2020). The different *Aeromonas* species present distinct pathogenic potentials, as a consequence of abundance and diversity of virulence genes, with *A. hydrophila* normally being associated with higher pathogenicity (Ghatak et al. 2016).

Despite the effect bacterial pathogens might have in Iberian leuciscids, deducing host-pathogen interactions based on mortality assessments is challenging. Detecting fish mortality in the wild can be impaired by ecosystem dynamics. Dead animals are rapidly eliminated from the habitat by predators and, unless mass mortality events occur (Vardakas et al. 2017), periodic surveillance strategies can fail to recognize the majority of cases. The development of active sampling schemes based on subsets of live individuals is a promising marker for wild fish population health's assessments. This strategy can allow to identify and categorize lesions' prevalence and severity in each population, as well as discriminate risk factors influencing it. Additionally, this type of program can likely be implemented across different regions and allow for results' comparison.

Susceptibility to bacterial pathogens in fishes varies both at the individual, populational and species level. In many cases, the observed trends are highly influenced by individuals' intrinsic determinants (Robinson et al. 2017). Infection challenge studies with different *Aeromonas* strains in cultured fishes exposed susceptibility/resistance patterns across different fish lineages and species (Fu et al. 2014, Zhou et al. 2017).

We hypothesize that sympatric species of Iberian leuciscids, sharing the same habitats, can be influenced at different degrees of severity by bacterial pathogens, even if exposed to similar environmental conditions, with potential consequences for individual survival. To test this, we conducted an exploratory survey with individuals from two non-migratory sympatric populations of leuciscids present in Portugal and evaluated individual skin lesion scores, *Aeromonas* species composition and strains' similarity and virulence profiles.

3.3. Materials and methods

3.3.1. Sampling site description and fish sampling

During field surveys in the dry season (June—October) of 2018 in the Lisbon area (Portugal), several cases of leuciscid individuals displaying skin lesions were registered. Lesion prevalence appeared to vary between species. *Iberochondrostoma lusitanicum* (Collares-Pereira 1980) and *Squalius pyrenaicus* (Günther 1868) individuals sharing the same habitat were selected for further analysis. Sampling occurred in October 2018 in the Jamor river (38.720832˚, -9.249696˚), a small coastal river basin located in an urban area, along a 30 m transect. Sampling location was selected based on previous knowledge of species co-occurrence (Sousa-Santos et al. 2016).

Fish were captured using standard electrofishing procedures (CEN 2003). Six individuals belonging to each species were randomly selected for analysis. After collection, animals were individually inspected for general status, their fork length was measured (length

from the tip of the snout to the notch of the caudal fin) and photographed from both lateral sides (Canon Digital Ixus 70 BKE). Animals were handled using protective material (i.e. nitrile gloves), skin was dried with a sterile gauze and a swab was performed along the body and caudal fin using an ESwab™ Liquid Amies Collection and Transport System (ThermoFisher Scientific, Massachusetts, USA). Body surface covered in the sampling process was similar for all individuals. Swabs were stored at 4˚C until further processing at the Laboratory of Microbiology and Immunology, Faculty of Veterinary Medicine, University of Lisbon, Portugal. All sampling was non-destructive, performed with manual immobilisation and animals were returned to the river after the procedure. Permits for fish capture were given by the competent authority (ICNF, permit number 477/2018/CAPT). All animals were cared for according to the rules given by the current EU (Directive 2010/63/EC) and national (DL 113/2013) legislation and by the competent authority (Direção Geral de Alimentação e Veterinária, DGAV, www.dgv.min-agricultura.pt/portal/page/portal/DGV) in Portugal. Only non-invasive samples were collected during routine procedures, and no ethics committee approval was needed. Trained veterinarians obtained all the samples, following standard routine procedures. No animal experiment has been performed in the scope of this research.

Water physical and chemical parameters were recorded. This included determination of pH, temperature, total dissolved solids and electrical conductivity; using a portable waterproof pH meter model HI98130 (Hanna Instruments, Rhode Island, USA); dissolved oxygen, using a waterproof oxygen meter model 9146–10, with probe HI76407/10F (Hanna Instruments, Rhode Island, USA); and nitrites and nitrates, using colorimetric strips (ITS Thorsten BetzelTM, Hattersheim, Germany).

3.3.2. Skin lesions' quantification

Photographs of lateral views (right and left) from each animal were used to analyse macroscopic morphology (i.e. ulcerations, haemorrhagic areas) of lesions in skin and produce an individual skin lesion score. Photographs were analysed by computer image software (ImageJ, Bethesda, Maryland, USA). The skin lesion score was calculated as follows: (total area of skin presenting lesions / total body area) x 100. Fins (except caudal fin) were excluded from the analysis since their visualization was not homogenous in the photographs. Scores were produced for both sides of the animal and an average score was obtained.

In order to differentiate two groups of individuals based on the extent of skin lesions, a division criterion was established based on the grouping characteristics of the observed data. Namely, two groups could be distinguished by observation based on a low prevalence of skin

lesions (skin lesion score lower than 2.5%) and a high prevalence of skin lesions (score higher than 5.4%). No score values between these thresholds were observed.

3.3.3. *Aeromonas* spp. quantification and isolation

Swabs were inoculated in tubes with 10 ml of Brain Heart Infusion (BHI) broth (VWR, Pennsylvania, USA), vortexed, after which serial ten-fold dilutions were performed in 9 ml of 0.9% saline solution (up to 10^{-4}). From each dilution (10^{-2} to 10^{-4}), 100 μl were inoculated in Glutamate Starch Red Phenol (GSP) Agar plates supplemented with 100,000 IU sodium penicillin g/l (Merck, New Jersey, USA), in duplicate. Plates were incubated at 37˚C for 12 h, for maximal identification probability of *Aeromonas* colonies through coloration. Bacterial quantification was performed for each plate and bacterial counts were averaged per individual (CFU/mL). GSP Agar is a selective and differential agar medium and *Aeromonas* spp. colonies are identified as large (2–3 mm), yellow and surrounded by a yellow zone. *Aeromonas hydrophila* ATCC 7966 was used as a positive control.

After incubation, for each individual fish sample, four single colonies of presumptive *Aeromonas* strains were randomly selected as previously described and further isolated into pure cultures in Brain Heart Infusion Agar (VWR, Pennsylvania, USA) for 24 h at 37 ˚C. Gram staining and oxidase activity of the isolates were evaluated. Isolates were stored in buffered peptone water (VWR, Pennsylvania, USA) with 20% glycerol at −80 ˚C during the study.

3.3.4. Molecular typing

Bacterial genomic DNA was obtained by the boiling method as described before (Talon et al. 1998). Molecular typing of the isolates was performed using a Random Amplified Polymorphic DNA (RAPD) method as previously described (Szczuka and Kaznowski 2004; Barroco 2013), with some modifications. Fingerprinting was achieved using the primers AP5 and AP3 (STABVIDA, Caparica, Portugal) (Szczuka and Kaznowski 2004) in independent mixtures.

Each amplification reaction was performed in a final volume of 25 μL, and the mixture consisted of 12.5 μL of Supreme NZYTaq 2× Green Master Mix (NZYTech, Lisbon, Portugal), 8.5 μL of PCR-grade water (Sigma-Aldrich, Missouri, USA), 0.5 μL (1 μM) of primer, 2.5 μL of Bovine Serum Albumine (0.01%; Thermo Fisher Scientific, Massachussets, USA) and 1 μL of template DNA (except for the negative control).

Thermocycler (VWR, Pennsylvania, USA) conditions included an initial step at 94 ˚C for 5 min; followed by 40 cycles of denaturation at 94 ˚C for 45 s, annealing at 40 ˚C for 1 min, and extension at 72 ˚C for 2 min; with a final extension step at 72 ˚C for 5 min.

Amplification products were resolved by agarose gel electrophoresis with 1.5% (w/v) agarose in 1X TBE Buffer (NZYTech, Lisbon, Portugal) for 50 min at 90 V. NZYDNA Ladder VII (NZYTech, Lisbon, Portugal) was used as a molecular weight marker. Gels were visualized using a UV light transilluminator and images recorded through the Bio-Rad ChemiDoc XRS imaging system (Bio-Rad Laboratories, California, USA).

3.3.5. *Aeromonas* spp. identification

Molecular species identification was performed by employing a multiplex PCR protocol previously described (Persson et al. 2015), with minor modifications. The established protocol targets *gyrB* and *rpoB* genes to discriminate between four *Aeromonas* species – *A. caviae*, *A. media*, *A. hydrophila* and *A. veronii*. As positive controls, *Aeromonas caviae* ATCC 1976, *Aeromonas hydrophila* ATCC 7966, *Aeromonas media* ATCC 33907 and *Aeromonas veronii* ATCC 35624 were used.

PCR mixtures were performed in a final volume of 25 µL and were composed of 12.15 µL of Supreme NZYTaq 2× Green Master Mix (NZYTech, Lisbon, Portugal), 10 µL of PCR-grade water (Sigma-Aldrich, Missouri, USA), 0.025 µL (0.05 µM) of primers A-16S, 0.25 µL (0.5 µM) of primers A-cav, 0.1 µL (0.2 µM) of primers A-med, 0.225 µL (0.45 µM) of primers A-hyd, 0.075 µL (0.15 µM) of primers A-Ver; and 1.5 µL of template DNA.

Thermocycler parameters were as follows: hot start at 95 ˚C for 2 min; followed by 6 cycles of denaturation at 94 ˚C for 40 s, annealing at 68 ˚C for 50 s, and extension at 72 ˚C for 40 s; and 30 cycles at 94 ˚C for 40 s, 66˚C for 50 s, and 72 ˚C for 40 s.

PCR products were resolved by agarose gel electrophoresis as previously described. Gels were resolved for 45 min at 90 V and NZYDNA Ladder VI (NZYTech, Lisbon, Portugal) was used as a molecular weight marker.

3.3.6. Virulence traits evaluation

In order to access the isolates' virulence phenotypes, different protocols previously described were employed with minor modifications. Namely, isolates were inoculated in Congo Red Agar (VWR, Pennsylvania, USA) for 72 h to detect the production of slime (Freeman et al. 1989), in Spirit Blue Agar (Difco, New Jersey, USA) supplemented with 0.2% Tween 80 (VWR, Pennsylvania, USA) and 20% olive oil (commercial) for 8 h for lipolytic

activity (Blaise and Armstrong 1973), in DNase Test Agar with Methyl Green (VWR, Pennsylvania, USA) for 24 h for DNase activity (Hickey et al. 2013), in Oxoid™ Nutrient Gelatin (Thermo Fisher Scientific, Massachussets, USA) for 24 h for gelatinase activity (Han et al. 2008), in Columbia agar supplemented with 5% sheep (VWR, Pennsylvania, USA) for 24 h for hemolytic activity (Santos et al. 1999), and in Skim Milk Agar (Sigma-Aldrich, Missouri, USA) for 24 h for proteolytic activity detection (Mellergaard 1983). Since fish are poikilothermic, incubation temperature was based on rivers' water temperature across the Lisbon district collected during dry season's field surveys (Sousa-Santos, unpublished data) in the period between 2017 and 2019 and averaged (22 ˚C).

The following strains were used as controls: *Aeromonas hydrophila* ATCC 7966 (DNase and haemolysin positive), *Aeromonas caviae* ATCC 15468 (haemolysin negative), *Escherichia coli* ATCC 25922 (DNase and gelatinase negative; slime non-producer), *Staphylococcus aureus* ATCC 29213 (lipase positive, protease negative), *Pseudomonas aeruginosa* Z25.1 clinical isolate from diabetic foot infection (protease and gelatinase positive; lipase negative), *Enterococcus faecium* EZ40 clinical isolate from canine periodontal disease (slime producer). *P. aeruginosa* and *E. faecium* (Mendes et al. 2012; Semedo-Lemsaddek et al. 2016) belong to the bacterial collection of the Laboratory of Microbiology and Immunology, Faculty of Veterinary Medicine, University of Lisbon, Portugal.

Virulence index was defined as the ratio between positive tests for virulence traits and the total amount of virulence traits evaluated and calculated for each isolate (Singh et al. 2017).

3.3.7. Data and statistical analysis

The reproducibility level of the genomic typing, molecular species identification and phenotypic virulence expression techniques was established by analysing a random sample of 10% replicates.

Genomic typing was carried out using BioNumerics1 version 7.6.3 software (Applied Maths, Sint-Martens-Latem, Belgium). Fingerprints similarity was obtained based on a dendrogram calculated with the Dice coefficient. A tolerance value of 1% was used for band matching. Cluster analysis was achieved through the unweighted pair group method with arithmetic average (UPGMA). The reproducibility value was determined as the average similarity value of all replicate's pairs (92.3%) and patterns with higher similarity values were considered undistinguishable.

Differences between the two fish species in 1) the skin lesion score, 2) the *Aeromonas* spp. quantification and 3) the mean virulence index per individual (mean across the isolates)

were evaluated using the Wilcoxon-Mann-Whitney test for independent samples. Association between the virulence index and 1) the species of *Aeromonas* and 2) the *Aeromonas*' cluster was evaluated using a generalized linear mixed model, with gamma as family and log link function, and with fish as a random effect (package lme4, version 1.1–10) (Bates et al. 2015). Correlation between the skin lesion score and 1) the individual size and 2) the *Aeromonas* spp. counts was determined using the Pearson correlation.

To understand if there was an association between skin lesion score and cluster I (more abundant and congregating most high skin lesion score's cases), a generalized linear model was used. Given the possibility of confounding that the fish species could have on this association, two models were used to differentiate the influence of this variable (with and without fish species). Since more than one bacterial isolate could be associated with each individual fish, a random sampling technique was used to generate correspondences between an isolate and an individual prior to the model implementation. This sampling technique was repeated 1000 times and the results were globally analysed regarding prevalence of occurrence. Effects were considered statistically significant when $p<0.05$. The statistical analysis was done using R software (R Core Team 2021). Graphs were produced using GraphPad Prism1 (GraphPad Software, San Diego, USA, version 5.01).

3.4. Results

3.4.1. Fish size, skin lesion score and water quality

While *I. lusitanicum* sampled set included both juveniles and adults (mean size=81.5 mm ± 18.2 SD; minimum-maximum range: 55–108 mm), we were only able to sample juvenile *S. pyrenaicus* in this study (60.2 mm ± 7.2; 49–69 mm). In general, *S. pyrenaicus* presented a higher degree of epidermic lesions, with the presence of variable areas of hyperaemic tissue and altered skin conformation (Figure 14).

Water level of the sampling location was considered normal for the seasonal expected levels and the stream presented a connected flow, allowing animals to perform movements along the habitat. Water physical and chemical parameters are displayed in Table 4.

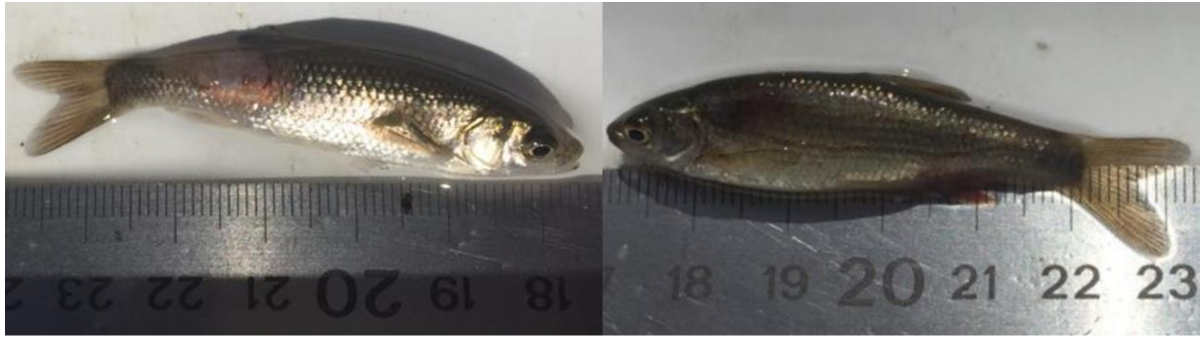


Figure 14. Examples of individuals collected during sampling. Left: *S. pyrenaicus* individual presenting an extensive area of epidermal loss with hyperaemia in the right dorso-lateral region of the pedunculus; Right: *I. lusitanicum* individual without skin lesions.

Table 4. Water physical and chemical parameters of the sampled stream.

Parameter	Value
pH	7.5
Temperature (˚C)	17.8
Dissolved Oxygen (ppm)	12.28
Total Dissolved Solids (ppm)	0.37
Electrical Conductivity (mS)	0.75
Nitrites (mg/l)	0.025
Nitrates (mg/l)	1

Skin lesions' prevalence varied significantly between the two species (p=0.015; Figure 15). The same did not occur for the *Aeromonas* spp. loads (p=0.589). No statistically significant association between the extent of the lesions and the size of the animal was observed (r=-0.531; p=0.076).

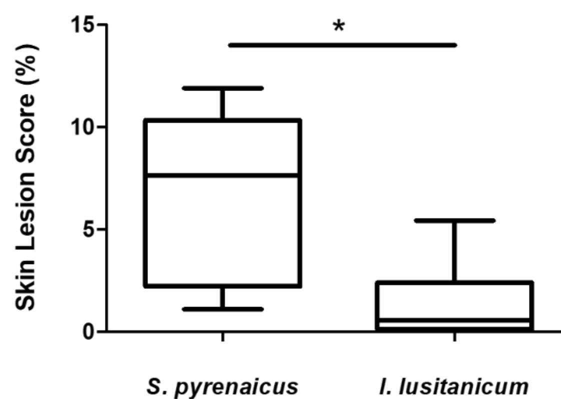


Figure 15. Skin lesion score (in %) in both analysed species. * p<0.05

3.4.2. *Aeromonas* load, identification and virulence index

Aeromonas isolation was possible from all individuals. Although no statistical differences were observed between the mean loads in both species ($\mu_{S.\ pyrenaicus}$=4.8x10^6 CFU/mL, $\mu_{I.\ lusitanicum}$=3.5x10^6 CFU/mL), different patterns were observed between individuals. No correlation between the skin lesion score and the *Aeromonas* spp. load was determined (r=-0.336; p=0.285).

The *Aeromonas* spp. diversity was different for each fish species and individuals (Figure 16). While *I. lusitanicum* appeared to present a higher number of bacterial species and respective proportions, a predominance of *A. hydrophila* was observed in the *S. pyrenaicus* individuals. Additionally, some *Aeromonas* species could only be detected in one of the fish species: *A. media* was only detected in *I. lusitanicum*, while *A. veronii* could only be isolated from *S. pyrenaicus*.

Figure 16. *Aeromonas* structure in *S. pyrenaicus* (left) and *I. lusitanicum* (right). Each line represents a sampled individual and shows the relative proportion of isolation (in %) of each *Aeromonas* species from the individual. Red–*A. hydrophila*, green–*A. caviae*, orange–*A. veronii*; blue–*A. media*.

Virulence index from isolates collected from both species differed significantly (p=0.009), with generally lower expression prevalence in *Aeromonas* spp. isolated from *I. lusitanicum*. The virulence index was also associated with the species of *Aeromonas* isolated. *A. hydrophila* and *A. veronii* presented significantly higher virulence index values (p<0.001) than *A. media* (Figure 17).

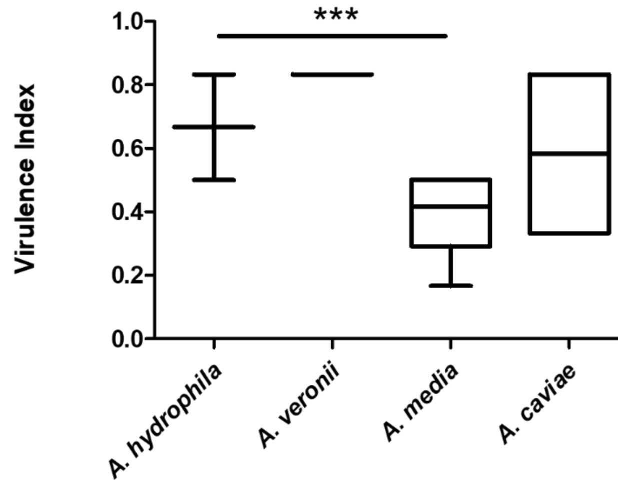


Figure 17. Virulence index by *Aeromonas* species. *** p<0.001.

Molecular typing of the isolates revealed seven clusters and two single member clusters (Figure 18), with a low prevalence of clones. Clones were isolated from the same animals.

Cluster I, determined at 42.2% similarity level, contains isolates identified as *A. hydrophila*, with the exception of E5.1 which was identified as *A. caviae*. The vast majority of the isolates belonging to cluster I were isolated from *S. pyrenaicus* and they concentrate the majority of animals displaying high levels of skin lesions. Cluster II, identified at 51.2% similarity, only included isolates from *I. lusitanicum*, encompassing different bacterial species (*A. hydrophila*, *A. caviae* and *A. media*) isolated from animals with different skin lesion scores. Cluster III, defined at 59.3% similarity, was formed by two *A. veronii* isolates originating from *S. pyrenaicus* with high levels of skin lesions. The remaining clusters were formed by isolates mainly from *I. lusitanicum* and animals with low scores. Cluster IV, with isolates both from *I. lusitanicum* and *S. pyrenaicus*, was defined at 50.7% and was exclusively composed by *A. hydrophila*. Cluster V, determined at 45.8%, was formed by *A. caviae* isolates from *I. lusitanicum*. Clusters VI and VII correspond to groups of *A. media* isolates from *I. lusitanicum*, determined at 48.8% and 58.3% similarity, respectively. Single member clusters corresponded to an *A. veronii* isolate from *I. lusitanicum* and an *A. hydrophila* isolate from *S. pyrenaicus*.

Aeromonas' cluster (single member clusters not considered) was significantly associated with the virulence index of the isolates, with members of clusters III and I presenting significantly higher index values (p<0.001) than members of clusters V and VII (Figure 19).

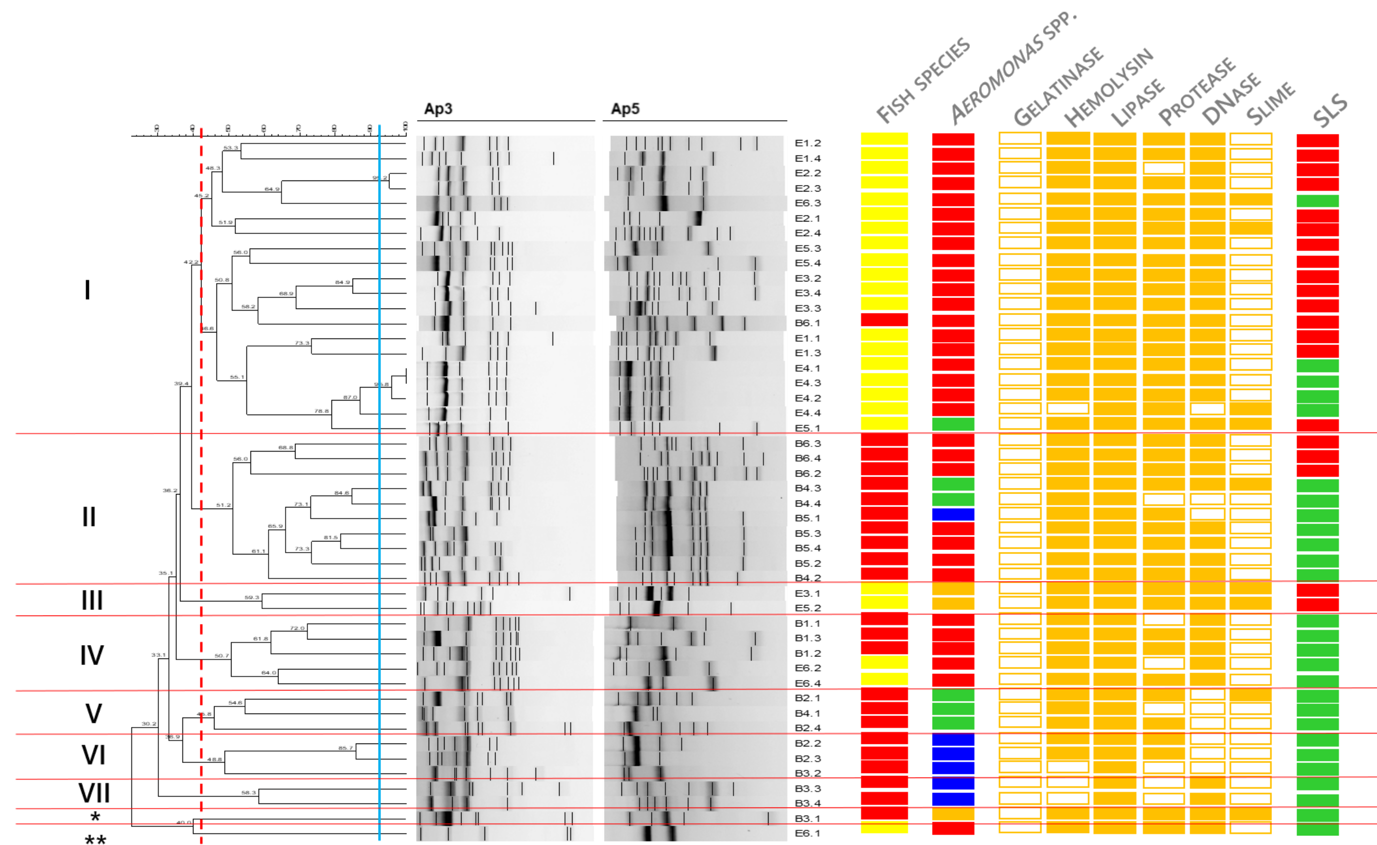


Figure 18. Dendrogram based on the composite analysis of the isolates' RAPD fingerprints with primers Ap5 and Ap3, using the Dice similarity coefficient. Clustering was achieved with UPGMA. Blue line represents reproducibility level (92.3%) and isolates displaying higher similarity levels were considered identical. Red dash line represents cluster level (42.2%). Red lines are presented for an easy visualization of the defined clusters. Cophenetic correlation coefficient was 0.74. First column represents isolate's identification, second the fish species (yellow–*S. pyrenaicus*, red–*I. lusitanicum*), third the *Aeromonas* species (red–*A. hydrophila*, green–*A. caviae*, blue–*A. media*, orange–*A. veronii*), fourth to ninth the virulence factors (gelatinase, hemolysin, lipase, protease, DNase, slime; empty rectangle–negative, full rectangle–positive) and the tenth the skin lesion score (SLS) [red–high (>5.4%), green–low (>2.5%)].

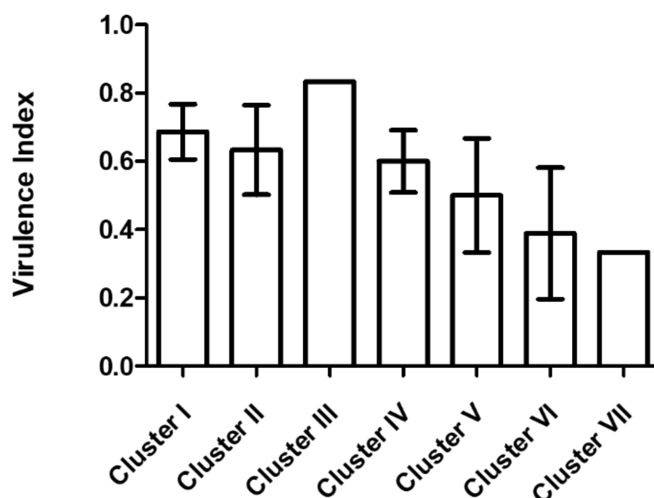


Figure 19. Virulence index by bacterial cluster.

3.4.3. Association between *Aeromonas* clusters and skin lesion score

Regarding the association between Cluster I and the skin lesion score, 80% of the iterations resulted in a significant association ($p<0.05$). However, such effect was not present when taking into account the fish species into the model (only 10% of the iterations resulted in $p<0.05$). This is a consequence of the confounding effect between the variables: in fact, all but one instance of cluster I were detected in *S. pyrenaicus*.

3.5. Discussion

Health assessments in Iberian leuciscids, apart from generally missing in conservation projects, can be challenging to achieve and to result in robust evidence of species susceptibility to particular threats. Active surveillance schemes can help to mitigate knowledge gaps in terms of particular species' tolerance to pathogens, important in conservation management planning. In this study, we show differences in skin lesions' prevalence among two sympatric Iberian leuciscid species and suggest a link between this observation and specific clusters of *Aeromonas* strains. Our results point to the existence of distinct susceptibility patterns from threatened Iberian leuciscid to this bacterial genus, both at individual- and species-level.

Fish sampled in this study showed some variation in terms of mean size, however, this is likely due to the fact that size classes might be distinctly represented for stochastic reasons and also due to the fact that the two species might show different and non-overlapping ecological requirements over the year and along their life span. Indeed, it is known that *I.*

almacai and *S. aradensis* (congeners of the target species in the present study) occupy distinct microhabitats throughout the year, overlapping only in the summer (Santos and Ferreira 2008). Although larger individuals from both species tend to select deeper and sheltered habitats, when transitioning from late summer to autumn, *I. almacai* shifts to shallower water streams. Juvenile fish, on the other hand, tend to accumulate in more exposed habitats along the year (Santos and Ferreira 2008), such as the location selected for this study's sampling. Additionally, an age-class distribution study in *Squalius laietanus* demonstrated a significant reduction of larger individuals in intermittent streams compared to smaller individuals (Merciai et al. 2017). This observation is similar to field annual census findings by our team (Sousa-Santos, unpublished data).

In cultured fish, it has been shown that juvenile stages often display higher mortality, or display more severe forms of disease, when challenged with *Aeromonas* spp. (Hawke and Khoo 2004). This feature is mainly attributed to an impaired immune function in early stages (Castro et al. 2015). Despite not statistically significant, our data suggests that the skin lesion score might be correlated with the size of the animals. However, it also suggests that there is an inverse trend between size and the levels of skin lesion in the fishes. To clarify this possible negative correlation, sampling of both juveniles and adults of *S. pyrenaicus* would be required. It would constitute a great advantage to sample both juvenile and adults of both species in order to discriminate the role of life stage on the susceptibility to *Aeromonas* species.

Physical and chemical water parameters did not differ significantly from those collected in previous years during annual census sampling (Sousa-Santos, unpublished data). However, some water parameters were considered to lay off the reference values. In farmed freshwater species, suggested nitrite and nitrate levels in the water should be undetectable (Noga 2010). Susceptibility to nitrite and nitrate poisoning is, nevertheless, species dependant and difficult to establish in wild Iberian leuciscids with current data. It is important to state that clinical signs of *Aeromonas* infection in fish are often the result of environmental stress (Beaz-Hidalgo and Figueras 2013). Poor water quality is often the origin of outbreaks in farmed species (El-Gohary et al. 2020). Additionally, mesophilic *Aeromonas* species' abundance is interconnected with environmental temperature and their levels are considered higher in summer months when water temperature increases (Lee et al. 2002). In modelling experiments regarding anthropogenic pressures, both *S. pyrenaicus* and *I. lusitanicum* are considered to display an intermediate tolerance to environmental alterations (Segurado et al. 2011). Annual censuses by our team (Sousa-Santos, unpublished data) suggests that *S. pyrenaicus* is an indicator species of good habitat quality, disappearing from streams showing initial signs of water quality detriment. Present results may indicate the effects of dry season

at the habitat level can have in these species; however, a comparison between different seasons and habitats with variable water quality is urged.

Previous studies in farmed species hinted at a correlation between *Aeromonas* spp. load and effects on the host, where an increase in the bacterial load results in magnified deleterious effects at the host level (i.e., morbidity and/or mortality) (Samayanpaulraj et al. 2019). However, our results do not evidence such relationship. A possible explanation relies on the evolution of host-microorganism interactions (Mikonranta et al. 2015). As an ubiquitous bacterial genus in aquatic environments, it is likely that Iberian leuciscids have been evolving in close contact with *Aeromonas* species. While some members of this genus may be more virulent than others, hosts will likely continuously evolve resistance trough immune activation which often results in decreases in bacterial load (Bonneaud et al 2019). So, observed results are possibly the mirror of an evolutionary arms-race between host and microbiota, with variable loads of *Aeromonas* spp. displayed by *S. pyrenaicus* individuals with higher levels of skin lesions.

Different bacterial diversities were isolated from the studied fish species. Several factors are involved in the determination of a host-microbiota composition. However, some factors seem to play a more determinant role (Smith et al. 2015). Steury et al. (2019) concluded that host population genetic divergence was more important in defining the gut microbiome of *Gasterosteus aculeatus* wild populations than environmental factors or the geographical area of origin. In our study, habitat sharing foresees a similar effect of environmental factors upon both species. Hence, an underlying host genotype difference could be the basis for the differential bacterial composition found.

The virulence index differed between isolates from *S. pyrenaicus* and *I. lusitanicum*. Since isolate structure in both fish species showed different *Aeromonas* species composition and respective clusters, virulence index values difference likely mirrors the differences between both fish species. An association was observed between the virulence index and the *Aeromonas*' cluster, demonstrating different virulence levels between clusters. *Aeromonas* spp. pathogenic potential can be the result of the presence of several virulence factors (Rasmussen-Ivey et al. 2016a). The virulence level differs among members of the genus *Aeromonas*, as proven by distinct genetic pools of virulence genes among species (Zhong et al 2019). Different pan-genome analysis showed this hierarchical pathogenicity among species, with *A. hydrophila* generally related with a higher pathogenic potential when compared to other species (Ghatak et al. 2016; Zhong et al. 2019). This hierarchical relationship is observed in our results, where clusters composed by this species were related to higher virulence indexes.

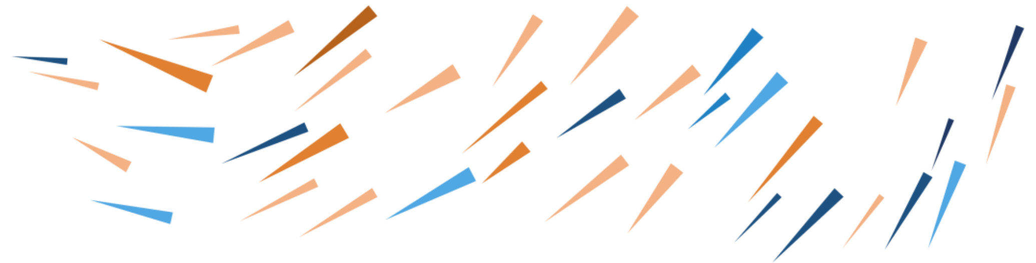
RAPD analysis was revealed as a good typing technique to differentiate the bacterial collection under study. Furthermore, this methodology allowed a fair differentiation of the bacterial clusters between two criteria: fish species and skin lesion score. Typing revealed that clusters I and III encompassed the majority of isolates originating from animals with high skin lesion scores. Additionally, isolates from these clusters also displayed the higher virulence indexes.

In situations of epidemics among a population it is common to observe a predominance of determined strains/clonal structures, often with a higher virulence profile. This situation was already documented in *Aeromonas* spp. outbreaks in cultured fish in North America and Asia (Rahman et al. 2002; Hossain et al. 2014; Rasmussen-Ivey et al. 2016b). In our study, however, it was not possible to discriminate if the cases of higher skin lesion scores were associated with the prevalence of a specific bacterial cluster or the species of fish. It is probable, though, that both variables are interconnected– i.e., *S. pyrenaicus* individuals likely present specific characteristics that make them more susceptible to the colonization and invasion by members of more virulent *Aeromonas* strains (e.g., clusters I and III) and that result in more pronounced alterations in the skin conformation. However, a sampling strategy with a higher number of individuals is needed in order to clarify this situation.

3.6. Conclusions

Current results shed light on the epidemiology of *Aeromonas* spp. in wild endangered leuciscids and suggest potential differences in susceptibility between different species/individuals. It is important to notice that species inhabiting the same geographical area and influenced by similar environmental pressures can harbour distinct bacterial compositions, exposing species-traits on a host-microbiome structure with potential impacts at the health level. Furthermore, we highlight the use of non-destructive technique in this investigation, stressing the importance of following similar methodologies across sampling schemes with threatened species.

Future studies in the field of bacterial infections and susceptibility in wild endangered fish species are needed. Future perspectives should include the comparison between species/populations exposed to distinct environmental conditions in order to disclose drivers of bacterial disease manifestation, as well as to investigate the genetic basis of susceptibility differences among species/populations, such as polymorphisms in the major histocompatibility complex, as a way to produce suitable markers of disease resistance to be used in conservation programs.



Chapter 4. *Aeromonas* spp. prevalence, virulence, and antimicrobial resistance in an *ex situ* program for threatened freshwater fish – A pilot study with protective measures

Grilo ML, Amaro G, Chambel L, Marques CS, Marques TA, Gil F, Sousa-Santos C, Robalo JI, Oliveira M. 2022. *Animals*. 12(4):436. doi: 10.3390/ani12040436.

Conceptualization, MLG, FG, CSS, JIR and MO; methodology, MLG, GA, LC, FG, CSS; software, MLG, LC, CSM and TAM; validation, MLG, GA, LC, CSM and TAM; formal analysis, MLG, LC, CSM and TAM; investigation, MLG and GA; resources, MLG and MO; data curation, MLG; writing—original draft preparation, MLG; writing—review and editing, MLG, GA, LC, FG, CSM, TAM, CSS, JIR and MO; visualization, MLG; supervision, JIR and MO; project administration, MO; funding acquisition, MLG, JIR and MO.

4.1. Abstract

Ex situ breeding programs are important conservation tools for endangered freshwater fish. However, developing husbandry techniques that decrease the likelihood of disease, antimicrobial resistance and virulence determinants acquisition during this process is challenging. In this pilot study, we conducted a captivity experiment with Portuguese nase (*Iberochondrostoma lusitanicum*), a critically endangered leuciscid species, to investigate the influence of simple protective measures (i.e., material disinfection protocols and animal handling with gloves) on the dynamics of a potential pathogenic genus, *Aeromonas*, as well as its virulence profiles and antimicrobial resistance signatures. Our findings show that antimicrobial resistance in *Aeromonas* spp. collected from *I. lusitanicum* significantly increased during the extent of the assay (5 weeks), with all isolates collected at the end of the study classified as multidrug-resistant. Additionally, humans handling fishes without protective measures were colonized by *Aeromonas* spp. The use of protective measures suggested a decreasing trend in *Aeromonas* spp. prevalence in *I. lusitanicum*, while bacterial isolates displayed significantly lower virulence index values when virulence phenotypical expression was tested at 22 °C. Despite this study representing an initial trial, which needs support from further research, protective measures tested are considered a simple tool to be applied in *ex situ* breeding programs for aquatic animals worldwide. Furthermore, current results raise concern regarding antimicrobial resistance amplification and zoonotic transmission of *Aeromonas* spp. in aquatic *ex situ* programs.

4.2. Introduction

Ex situ breeding and recovery programs are important conservation tools that help to secure species experiencing severe declines in their natural habitat (McGowan et al. 2017). In Portugal, a dedicated *ex situ* breeding program was established in 2008 aiming to counteract the threatened status of endemic leuciscid species (Sousa-Santos et al. 2014b). This group of freshwater fishes, presenting a high level of endemicity in the Iberian Peninsula, faces severe conservation constraints associated with habitat degradation, water extraction, summer droughts, and the proliferation of invasive species (Sousa-Santos et al. 2014b). Despite the conservational value of *ex situ* programs for wild species, these actions are not met without a cost. The translocation of species from their original habitat into anthropogenic facilities, along with the changes implemented in the husbandry of the individuals during their stay in the program and inherent changes in phenotypical and genotypical traits of the animals caused by captivity, are accompanied by important modifications in the animal's microbiota (McKenzie et al. 2017). Changes in the host microbiota composition and traits can impair overall fitness and present severe consequences for the individual survival (Bates et al. 2019).

Additionally, and since dissemination of antimicrobial resistance and virulence determinants between different environments are a general public health concern, the role of recovery and breeding programs as gateways of antimicrobial resistance and virulence transfer between anthropogenic cycles and natural environments needs to be addressed in the One Health context. Previous studies have addressed the effect that recovery programs have had in the acquisition of resistance determinants by wild animals in close contact with humans (Stoddard et al. 2009; Power et al. 2013). However, no study so far has explored such dynamics in ex situ conservation programs with aquatic species. The relocation of these animals into their natural habitats may establish new communication bridges and the formation of additional resistance and virulence determinants reservoirs in natural environments, which are difficult to be controlled and eradicated.

It is fundamental to understand current reality and adapt captivity's conditions and husbandry techniques in order to minimize alterations in the host microbiota during the program extent, as well as to prevent the acquisition and further dissemination of resistance and virulence determinants that can constitute reservoirs when released into the wild.

In this pilot study, in order to understand the dynamics of prevalence and antimicrobial resistance and virulence determinants of *Aeromonas* spp., an important zoonotic and fish pathogenic agent, we developed a captivity experiment with Portuguese nase (*Iberochondrostoma lusitanicum*) individuals under two husbandry regimens with different biosafety measures and evaluated the prevalence and structure of *Aeromonas* spp., their antimicrobial resistance signatures, and virulence profiles.

4.3. Materials and Methods

4.3.1. *I. lusitanicum* capture and transport

The *ex situ* conservation program, ongoing at the Vasco da Gama Aquarium (Lisbon, Portugal), is responsible for the captive breeding, for restocking purposes, of five threatened leuciscids (*Achondrostoma occidentale*, *Anaecypris hispanica*, *Squalius pyrenaicus*, *Iberochondrostoma almacai* and *Iberochondrostoma lusitanicum*). Populations of each species considered to be at higher risk are selected to be included in the program and a stock of wild adults is collected from the natural habitat and housed in separate tanks to prevent contact.

Due to space limitation in the program, selection of populations for breeding is performed through a rotation scheme. This study benefited from the fact that a new wild stock of *I. lusitanicum* from the Sado river was going to be established at the Vasco da Gama Aquarium facilities. Hence, this species was selected to participate in this study.

Fishing and detention licenses were granted by the competent authority - Instituto da Conservação da Natureza e das Florestas, IP (Fishing Credential nº 71-A/2018; License nº 438/18/CAPT). The animals included in this study were cared for according to the rules given by the current EU (Directive 2010/63/EC) and national (DL 113/2013) legislation and by the competent Portuguese authority (Direção Geral de Alimentação e Veterinária, DGAV, www.dgv.min-agricultura.pt/portal/page/portal/DGV). In the scope of this study, only non-invasive samples were collected during the routine procedures, and no ethics committee approval was needed. Samples were obtained by trained veterinarians, following standard routine procedures. No animal experiment has been performed in the scope of this study.

Animals were collected by electrofishing following standard procedures (CEN 2003). Captured fish were transferred to a container with water collected at the sampling site in order to monitor the stunning recuperation. The animals included in this study were collected at Ribeira de Grândola (38.168980°, -8.569030°) in May, 2018. In total, 22 adult individuals were collected (eight females, six males and eight undetermined; mean size = 73.9 mm ± 10.3 SD).

After capture, animals were placed in a transport container with water collected from the river stream. The container dimensions were appropriate for the expected animal abundance in order to guarantee the absence of casualties during transportation (50 L for 30 individuals). Animals were transported by car to Vasco da Gama Aquarium.

4.3.2. Experimental setup

In order to mimic the breeding program conditions, the experiment was set in the same area where the breeding tanks are usually located – an exterior terrace, under natural light, temperature and pluviosity at the Vasco da Gama Aquarium. Experimental conditions applied during this study are summarized in Table 5.

The experimental setting consisted of two 100 L tanks with closed water circulation and sand filtration systems (obtained from tanks already established). Two fish refuge made by PVC tubes were included in each tank. The tanks were disinfected with 70% ethanol prior to their filling with water. Circulation, filtration and refuge materials were immersed in 70% ethanol for 24 h, followed by periods of 15 min exposure to UV radiation of all their surfaces (the number of periods varied according to the number of surfaces of the object). Replicates of each condition (i.e., tanks) were not performed due to technical limitations (i.e., tank uniformity and availability) and sample size (i.e., low number of animals, gregarious behaviour of *I. lusitanicum*, stress effects due to group separation and possible interference with reproduction, high microbiota inter-variability among individuals).

Aeromonas spp. screening was performed from the following samples: swabs from the tanks' walls and floor after disinfection, food (frozen mosquito larvae, frozen *Mysis* shrimp and frozen krill), water samples from the aquarium supplier (directly from the tap, 36 h prior to tank filling) and filtering sand.

Tank preparation was initiated 120 h prior to fish introduction to allow the water to stabilize and to prevent shocks leading to fish mortality. Tank conditions differed regarding contact with the environment. In the control tank, a fine mesh (ca. 2 mm) plastic cover was used to cover the top of the tank, still allowing the entrance of external agents (i.e. avian faeces, insects, rain water, dust), as happens in the tanks currently used in the program. In the test tank, a plastic cover was used over the surface of the tank, restraining the entrance of external agents.

Table 5. Experimental conditions in this study.

Action	Control Tank	Test Tank
Tank covering	Fine mesh	Plastic cover
Food preparation	Food items thawed with tap water in non-disinfected containers	Food items thawed with sterile water transported in sterile shots in containers disinfected with 70% ethanol and exposed to UV radiation
Water renovation and food surplus retrieval	Water and food pumped out of the tank with a non-disinfected suction system	Water and food pumped out of the tank with a suction system disinfected with 70% ethanol and exposed to UV radiation
Fish handling	Operator not using gloves and using non-disinfected shrimp nets and handling tanks	Operator using nitrile gloves and handling tanks disinfected with 70% ethanol and shrimp nets disinfected with 70% ethanol and exposed to UV radiation
Fish sampling	Measuring device non-disinfected	Measuring device disinfected with 70% ethanol

The feeding regimen followed the protocol established for this species in captivity (Sousa-Santos et al. 2014b). Food sources were limited to frozen mosquito larvae, *Mysis* shrimp, and krill. Food was thawed two hours prior to feeding and cleaned with water to eliminate impurities. Food for animals at the control tank was cleaned with water directly from tap and stored in current use containers (not exposed to disinfection protocols), while food for the animals at the test tank was cleaned with sterile water stored at sterile shots and stored in containers disinfected with 70% ethanol and exposed to UV radiation.

Water renovation was performed weekly and consisted of the extraction of 10 L of tank water and slow introduction of the same amount of tap water.

Handling of animals, food and water from the control tank was performed by two rotating aquarists not subjected to disinfection protocols. Any action directed towards the test tank was performed by an external specialized operator, using nitrile gloves disinfected with 70% ethanol. Bacterial sampling was performed weekly on a fasting day. Sampling materials used in the test tank (shrimp net and water suction system) were exposed to UV radiation. Materials used in the control tank were not disinfected.

By the end of the assay, animals were introduced in the breeding tanks of the *ex situ* conservation program.

4.3.3. Assay with distinct husbandry protocols

A weekly scheme of the assay, including tasks performed on each day, is displayed in Table 6. The assay was performed for 5 weeks.

Table 6 Operation scheme of the assay.

Week	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
1st	Capture + Transport 1st Bacterial Sampling	Fasting	Feeding Food Sampling	Water Renovation Water Sampling	Feeding	Fasting	Fasting
2nd	Feeding	2nd Bacterial Sampling	Feeding Food Sampling	Water Renovation Water Sampling	Feeding	Fasting	Fasting
3rd	Feeding	3rd Bacterial Sampling	Feeding Food Sampling	Water Renovation Water Sampling	Feeding	Fasting	Fasting
4th	Feeding	4th Bacterial Sampling	Feeding Food Sampling	Water Renovation Water Sampling	Feeding	Fasting	Fasting
5th	Feeding	5th Bacterial Sampling					

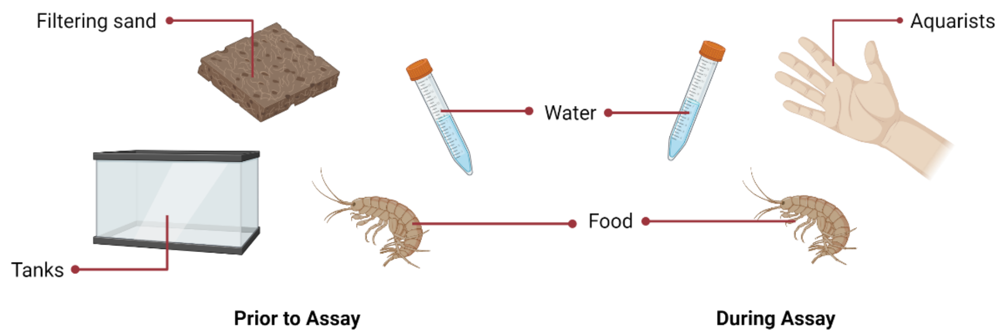
Following arrival to Vasco da Gama Aquarium, the animals were randomly selected and divided in two groups (n=11, each; test group—mean size =72.5 mm ± 10 SD, control group—mean size =75.3 mm ± 10.4 SD). Each animal was measured, sex determined (when possible), inspected for diagnostic phenotypic individual traits and a body swab (cloacal area, lateral sides of the body and fins, excluding head; ESwab LiquidAmies Collection and Transport System, ThermoFisher Scientific, Massachusetts, USA) was collected to establish an initial bacteriological baseline. Afterwards, animals were introduced to the corresponding tank.

In each bacteriological sampling action, water from each tank was transferred to a separate tank, followed by the transfer of the individuals collected with shrimp nets. Animals were individually measured, a swab sample was performed, and the animals were returned to their respective tanks. Different measuring devices and handling tanks were used between individuals from different tanks to prevent cross-contamination (Figure 20).

In the control tank, the aquarist did not use gloves or disinfect their hands before handling the animals. From the 3rd to the 5th bacteriological sampling action in the control tank, both prior to and after the procedures, a swab was collected from the hands, fingernails, and lower arms of the aquarist. Human sampling was performed after the individuals were informed regarding the sampling procedure, and signed an informed consent in accordance with the Helsinki Declaration of the World Medical Association (version October 2013) and the Oviedo Convention (version April 1997). No human experiment has been performed in the scope of this study.

Food and water samples were collected weekly, at regular days, by using sterile 50 mL tubes (Corning Life Sciences, New York, NY, USA).

Water quality was controlled over the experiment's period by determination of a set of physical and chemical parameters: pH (mean = 7.02 ± 0.5 SD) and temperature (mean = 14.9 °C ± 0.8 SD), using a portable waterproof pH meter model HI98130 (Hanna Instruments®, Woonsocket, RI, USA); nitrites (mean = 0 mg/L ± 0 SD) and nitrates (mean = 0 mg/L ± 0 SD), using colorimetric strips (ITS Thorsten BetzelTM, Hattersheim, Germany).



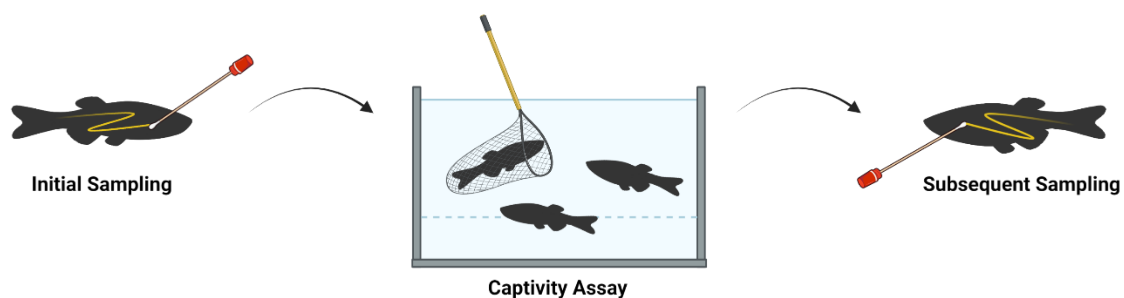


Figure 20. Sampling scheme for *Aeromonas* detection. Material used in the experiment that were not possible to disinfect through UV radiation were sampled priorly to the beginning of the assay (tanks and filtering sand), as well as the frozen food items used during the assay and the water used to fill the tanks. During the assay, weekly sampling of food items (after tawing and before introduction in the aquarium) and water (directly collected from the tanks by total immersion), as well as swabs collected from the hands and arms of the aquarists working on the control tank (from 3rd to 5th sampling week, prior and after contact to fishes and water) were performed. Fish were sampled after collection in the wild and prior introduction in the respective tank. After, weekly sampling actions were performed for each individual. Created with BioRender.com

4.3.4. Aeromonas spp. isolation, genomic typing and identification

Selection of *Aeromonas* spp. as model pathogens was based on their relevance as fish pathogens. *Aeromonas* spp. are one of the most common pathogen groups of freshwater fishes, and although their impact in Iberian leuciscid populations is unknow, their impact in both wild and farmed fishes is emphasized, especially in stressful conditions such as the translocation into new environments (Cipriano 2001).

After collection, swabs (both from human, animal and food samples) were inoculated in tubes with 8 ml of Brain Heart Infusion (BHI) broth (VWR, Pennsylvania, USA), subjected to homogenization, and incubated at 37 °C for 24 h. Regarding water samples, tubes

containing the samples were homogenized briefly and 100 µL of each sample were transferred into BHI broth, followed by a similar protocol to the one applied to the rest of the samples. Following incubation, a sample from each tube was transferred to Glutamate Starch Red Phenol (GSP) Agar plates supplemented with 100,000 IU sodium penicillin g/L (Merck, New Jersey, USA). This is a selective and differential agar medium for *Aeromonas* spp., and typical colonies are large (2-3 mm), yellow and surrounded by a yellow zone. Plates were incubated at 37 °C for 12 h. Four distinct colonies displaying *Aeromonas* spp. morphology were randomly selected from each individual sample and isolated into pure cultures in BHI agar, at 37 °C for 24 h. *Aeromonas hydrophila* ATCC 7966 was used as a positive control.

Isolates were characterized regarding Gram-staining and oxidase activity. Pure cultures of oxidase positive gram-negative rods were stored in buffered peptone water (VWR, Pennsylvania, USA) with 20% glycerol at −80 °C during the study.

Bacterial genomic DNA was obtained by the boiling method, after growth in BHI agar (37 °C, 24 h), as described before (Talon et al. 1998).

In order to perform the molecular typing of the isolates, a Random Amplified Polymorphic DNA (RAPD) technique was used. The method was applied as described before (Szczuka and Kaznowski 2004; Barroco 2013), with minor modifications. Primers Ap3 and Ap5 (Szczuka and Kaznowski 2004) were chosen and used in independent mixtures to achieve fingerprinting patterns of the isolates. Each amplification reaction was performed in a final volume of 25 µL. The mixture consisted of: 12.5 µL of Supreme NZYTaq 2× Green Master Mix (NZYTech, Lisbon, Portugal), 8.5 µL of PCR-grade water (Sigma-Aldrich, Missouri, USA), 2.5 µL of Bovine Serum Albumine (0.01%; Thermo Fisher Scientific, Massachussets, USA), 0.5 µL (1 µM) of primer and 1 µL of template DNA. Thermocycler conditions used included 94 °C for 5 min; 40 cycles of 94 °C for 45 s, 40 °C for 1 min, and 72 °C for 2 min; and 72 °C for 5 min.

PCR products were resolved by gel electrophoresis [1.5% (w/v) agarose in 1X TBE Buffer (NZYTech, Lisbon, Portugal)] for 50 min at 90 V. As a molecular weight marker, NZYDNA Ladder VII (NZYTech, Lisbon, Portugal) was used. The visualization of gels was performed using a UV light transilluminator. Images were captured using the Bio-Rad ChemiDoc XRS imaging system (Bio-Rad Laboratories, California, USA).

In order to achieve species identification, a multiplex PCR protocol, previously described by Persson et al. (2015), was used with minor modifications. This protocol discriminates between *A. caviae*, *A. media*, *A. hydrophila* and *A. veronii*, based on *gyrB* and *rpoB* genes' sequences, and contains an internal control for the genus *Aeromonas*. PCR mixtures were performed in a final volume of 25 µL and were composed of 12.15 µL of

Supreme NZYTaq 2× Green Master Mix (NZYTech, Lisbon, Portugal), 10 µL of PCR-grade water (Sigma-Aldrich, Missouri, USA), 0.025 µL (0.05 µM) of primers A-16S, 0.25 µL (0.5 µM) of primers A-cav, 0.1 µL (0.2 µM) of primers A-med, 0.225 µL (0.45 µM) of primers A-hyd, 0.075 µL (0.15 µM) of primers A-Ver, and 1.5 µL of template DNA. Thermocycler (VWR, Pennsylvania, USA) conditions were as follow: 95 °C for 2 min, followed by 6 cycles of 94 °C for 40 s, 68 °C for 50 s and 72 °C for 40 s; and 30 cycles at 94 °C for 40 s, 66 °C for 50 s, and 72 °C for 40 s. *A. caviae* ATCC 1976, *A. hydrophila* ATCC 7966, *A. media* ATCC 33907 and *A. veronii* ATCC 35624 were used as positive controls.

Amplification products were resolved by gel electrophoresis and visualized as described above. Gels were resolved for 45 min at 90 V. As a molecular weight marker, NZYDNA Ladder VI (NZYTech, Lisbon, Portugal) was used.

4.3.5. Virulence factors screening

The virulence factor expression by the isolates was accessed by a set of phenotypical assays, as established in protocols previously described, with minor modifications. Screening was performed on isolates collected during the first and the fifth sampling week. The following virulence factors were investigated, namely: 1) gelatinolytic activity, using Oxoid Nutrient Gelatin (Thermo Fisher Scientific, Massachussets, USA) for 24 h (Han et al. 2008), 2) hemolytic activity, using Columbia agar supplemented with 5% sheep blood (VWR, Pennsylvania, USA) for 24 h (Santos et al. 1999), 3) lipolytic activity, using Spirit Blue Agar (Difco, New Jersey, USA) supplemented with 0.2% Tween 80 (VWR, Pennsylvania, USA) and 20% olive oil (commercial) for 8 h (Blaise e Armstrong 1973), 4) proteolytic activity, using Skim Milk Agar (Sigma-Aldrich, Missouri, USA) for 24 h (Mellergaard 1983), and 5) slime production, using Congo Red Agar for 72 h (Freeman et al. 1989). Two incubation temperatures were used. The 22 °C was an average based on river's water temperature data collected during annual monitoring census performed in the summer seasons of 2017 to 2019 (Sousa-Santos, personal communication). This was performed in order to mimic fish's body temperature since they are poikilothermic. The 37 °C was used to mimic human body's temperature.

The following strains were used as controls: *A. caviae* ATCC 15468 (hemolysin negative), *A. hydrophila* ATCC 7966 (hemolysin positive), *Enterococcus faecium* EZ40 clinical isolate canine periodontal disease (slime producer), *Escherichia coli* ATCC 25922 (gelatinase negative; slime non-producer), *Pseudomonas aeruginosa* Z25.1 clinical isolate diabetic foot infection (protease and gelatinase positive; lipase negative) and *Staphylococcus aureus* ATCC 29213 (lipase positive, protease negative). *P. aeruginosa* and *E. faecium* (Mendes et al. 2012; Semedo-Lemsaddek et al. 2016) belong to the bacterial collection of the Laboratory

of Microbiology and Immunology, Faculty of Veterinary Medicine, University of Lisbon, Portugal.

The virulence index of each isolate was calculated based on the ratio between positive tests for virulence factors and the total amount of virulence factors tested (Singh et al. 2017).

4.3.6. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the disk diffusion technique (Bauer et al. 1966). Guidelines and breakpoints of the Clinical and Laboratory Standards Institute were followed as reference (NCCLS 2002; CLSI 2013). The following antibiotics (Mastdiscs, Mast Group, Liverpool, United Kingdom) were tested: amikacin (AK, 30 µg), amoxicillin/clavulanic acid (AUG, 20-10 µg), aztreonam (ATM, 30 µg), ceftazidime (CAZ, 30 µg), enrofloxacin (ENF, 5 µg), erythromycin (E, 15 µg), florfenicol (FFC, 30 µg), imipenem (IMI, 10 µg), nitrofurantoin (NI, 300 µg), streptomycin (S, 10 µg), tetracycline (T, 30 µg) and sulfamethoxazole/trimethoprim (TS, 23.75-1.25 µg). Antimicrobial compound choice followed those commonly used to treat Gram-negative infections in Human and Veterinary Medicine, as well as those compounds used for treating aquatic animals' diseases. *Escherichia coli* ATCC 25922 was used as a quality control.

Isolates were categorized as multidrug-resistant, as described by Magiorakos et al. (2012), when presenting non-susceptibility to at least one antimicrobial compound in three or more antimicrobial categories. Multiple antibiotic resistance (MAR) index values were produced for each isolate and calculated based on the ratio between the number of antimicrobial compounds to each the isolate presenting a non-susceptibility profile and the total amount of antimicrobial compounds tested (Krumperman 1983). Non-susceptibility was defined as presenting intermediate or resistant category status. Antimicrobial compounds to which *Aeromonas* spp. are considered intrinsically resistant (amoxicillin/clavulanic, erythromycin and streptomycin) were not included in the multidrug resistance characterization and in the MAR index calculation.

4.3.7. Statistical analysis

In order to analyse the reproducibility level of the molecular species identification, phenotypic virulence expression, antimicrobial susceptibility testing and genomic typing techniques, a random sample including 10% replicates was used.

BioNumerics version 7.6 software (Applied Maths, Sint-Martens-Latem, Belgium) was used to perform genomic typing. The similarity of the fingerprinting patterns was achieved

based on a dendrogram calculated with the Pearson correlation coefficient. Cluster analysis was performed through the unweighted pair group method with arithmetic average (UPGMA). The reproducibility value of the technique was determined as the average similarity value of all replicate's pairs (91.88%). When patterns presented higher similarity values, they were considered to be undistinguishable. Clusters were formed based on a joint evaluation of the fingerprinting profiles and the *Aeromonas* species. For all dendrograms and all clusters, the lowest similarity value was investigated and a cut off value was established across dendrograms to form clusters and enable comparisons between dendrograms

Several isolate level response variables were modelled as a function of tank and sampling week. At the isolate level, and therefore, using a GLMM (Generalized Linear Mixed-Effects Models) (Bates et al. 2015) with fish as a random effect, we modelled as a binomial logistic response the virulence factors (i.e., prevalence of individual activity of each of the tested virulence factors) at both (1) 22°C and (2) 37°C (0 – Negative, 1 – Positive) and the (3) categories of susceptibility to antibiotics (0- Non-susceptible, 1-Susceptible), and using a beta response (continuous values ranging from 0 to 1), the (4) MAR index values (i.e., the ratio between the number of antimicrobial compounds considered as presenting acquired non-susceptibility and the total amount of antimicrobial compounds tested) and the virulence index values (i.e., the ratio between the number of virulence factors for which positive activity was detected and the total amount of virulence factors tested) at both (5) 22°C and (6) 37°C.

Using a multinomial log-linear model (package nnet, version 7.3-15) (Venables and Ripley 2002), the (7) proportion of the different species of *Aeromonas* was considered.

Considering an analysis at the tank level, a GLM with a beta response was used to model the (8) Simpson index and the (9) prevalence of *Aeromonas* spp. The statistical analysis was done using R software (R Core Team 2021). Graphs were produced using GraphPad Prism (GraphPad Software, San Diego, USA, version 5.01).

4.4. Results

Mortality was not observed in both tanks during the extent of the assay. While individuals displayed a shy behaviour in the first two days of the experiment and remained in the refuges provided, swimming, exploration, and feeding behaviour was considered normal for the remaining period.

Aeromonas spp. isolation was achieved for all animals in the beginning of the trial. However, prevalence in each tank varied across the assay's weeks. While *Aeromonas* spp. prevalence in the control tank was of 100% or close to it in every week, prevalence in the test tank decreased to 54.5% in the last week of sampling, although it displayed similar prevalence

values to the control tank in the previous weeks (Figure 21). This variation was significantly different among tanks (p<0.001).

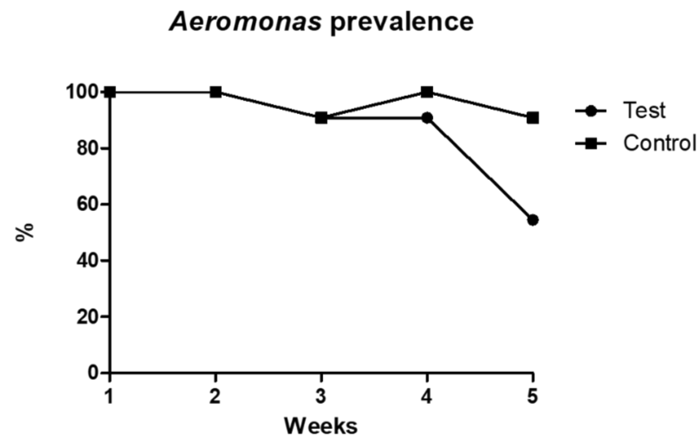


Figure 21. ***Aeromonas*** **spp. prevalence in both tanks across the sampling weeks.**

Prior to the beginning of the captivity assay, *Aeromonas* spp. were not detected in the evaluated matrixes (food, water and filtering sand). Similarly, no *Aeromonas* spp. were isolated from the water samples collected in both tanks across the assay. Regarding food sources, it was possible to detect *Aeromonas* spp. from the food administered in the control tank in the 1st and 2nd week of sampling. No *Aeromonas* spp. were isolated from the food available at the test tank. Regarding the samples collected from aquarists' hands and arms, all swabs obtained after handling the animals were positive for *Aeromonas* spp. However, none of the swabs collected prior to handling were positive for *Aeromonas* spp.

Initial *Aeromonas* species structures were similar between individuals from both tanks (Figure 22). Some shifts were observed (using a multinomial log-linear model) along the assay weeks regarding species prevalence between the two tanks. Although *A. veronii* isolation in the test tank was possible across almost all sampling actions, such phenomenon was not observed in the control tank. Instead, an increase of *A. media* prevalence was found from the first sampling action to the second, with presence also recorded in the last week of sampling. Interestingly, the original predominance of *A. veronii* in both tanks was gradually substituted by a predominance of *A. hydrophila* in both tanks. Both the sampling tank and week significantly influenced the *Aeromonas* species structure. *A. veronii* was more prevalent in the test tank (p=0.006) in comparison with the control tank, while an opposite trend was observed for *A. media* (p 0.050). *A. veronii* prevalence also significantly shifted since week one, decreasing in the second and the third weeks (p<0.001) and increasing in the final week (p<0.001).

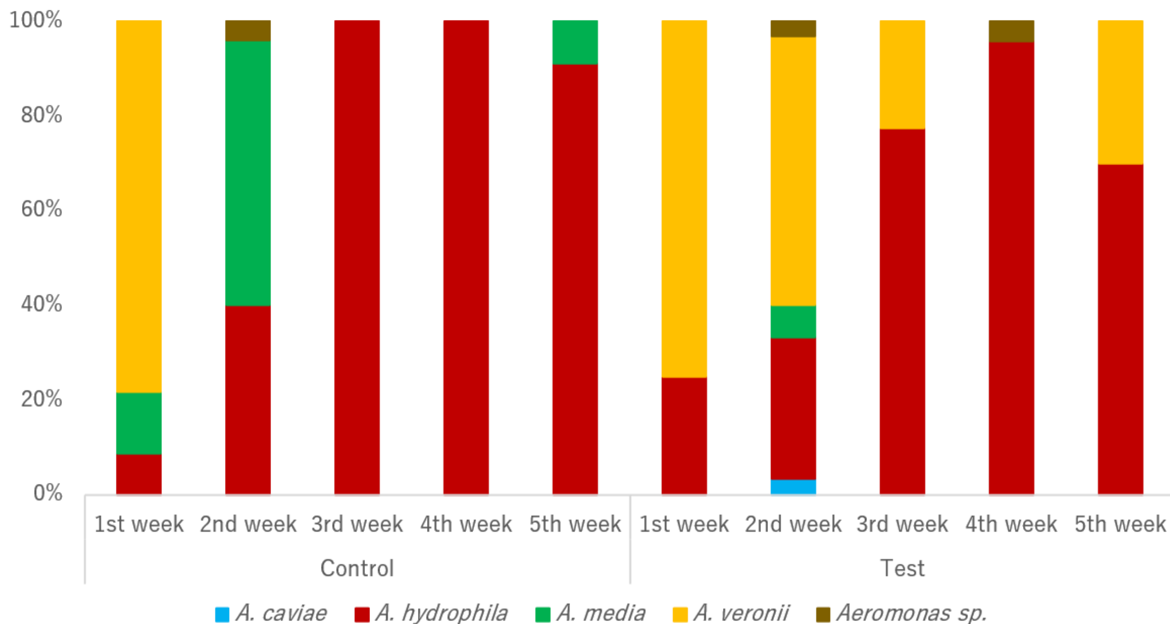


Figure 22. Relative prevalence of *Aeromonas* species by tank and sampling week.

Typing the 363 isolates and evaluating their relationships based on dendrogram analysis revealed the presence of 54 clusters and 40 single-member clusters. A cut off level of 62.93% was assumed based on the evaluation of fingerprinting patterns, *Aeromonas* species, tank of origin and sampling week (Supplementary Figure S16, Annex XVII). In both tanks, a total of 58 clones were identified at 91.88% similarity (reproducibility level). Around half of the clones detected were collected from the same individual, while the other half were collected from different individuals in the same tank and in the same sampling week. None of the clones were isolates collected from fishes in different tanks or across sampling weeks. The only observed exceptions were the food sources and the aquarists. All six isolates of *Aeromonas* sp. collected from the food were obtained from the first and the second week of sampling and were clones. Regarding the isolates collected from the aquarists' hands, two isolates from the same aquarist in the third and fourth sampling week were clones.

Although no specific pattern was observed for both tanks, some clusters were exclusive to a particular sampling week (Supplementary Figure S17 and S18, Annexes XVIII and XIX). Some clones were observed, namely clones of *A. veronii* isolated from the food sources, clones of *A. hydrophila* in the fourth week of sampling in the control tank, clones of *A. hydrophila* in the fourth week of sampling in the test tank, and clones of *A. hydrophila* in the fifth week of sampling in the test tank. Regarding the isolates collected in the same tank and in the same sampling week (Supplementary Figure S19, Annex XX), when strains of different *Aeromonas* species were detected, they formed distinct clusters.

When testing the virulence factor's phenotypic expression of the isolates at 22°C (Figure 23), the virulence index values obtained for the isolates in the test tank in the fifth week were significantly lower than the ones in the control tank and the ones recorded for the first sampling week (p=0.003). No differences were observed between the virulence indexes from isolates from both tanks (p=0.337) at each sampling week (p=0.580) when tested at 37°C.

The prevalence of virulence factors in isolates tested at 22°C differed among experimental conditions and sampling weeks (Figure 24). Regarding hemolytic activity, prevalence was significantly lower in the isolates from fifth week in comparison with the first one (p=0.050), with a decrease in the isolates from the control tank. Lipolytic activity wasn't influenced by the experimental condition (p=0.984) and sampling week (p=0.974). Gelatinolytic activity differed among weeks (p=0.040), being characterized by an increase in the isolates from the control tank and a decrease in the isolates from the test tank. Proteolytic activity was also influenced by the tank (p=0.021) and the sampling week (p=0.032), being significantly lower in the isolates from the test tank in the fifth week. Slime production was significantly different between experimental conditions (p=0.050), being lower in the isolates from the test tank. Regarding the prevalence of virulence factors tested at 37°C, the majority of the phenotypic traits did not present differences between the studied variables. The only exception was for the proteolytic activity that was significantly different between weeks (p=0.039), being lower in the isolates from the fifth week in the control tank.

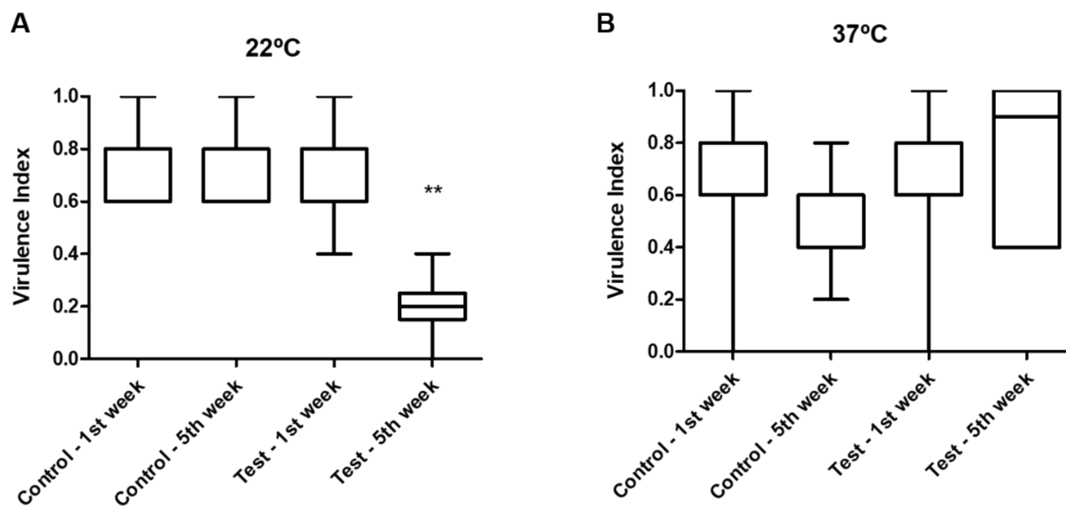


Figure 23. Virulence index of the isolates collected in both tanks in the 1st and 5th week and analysed at 22°C (A) and 37°C (B). ** p<0.01

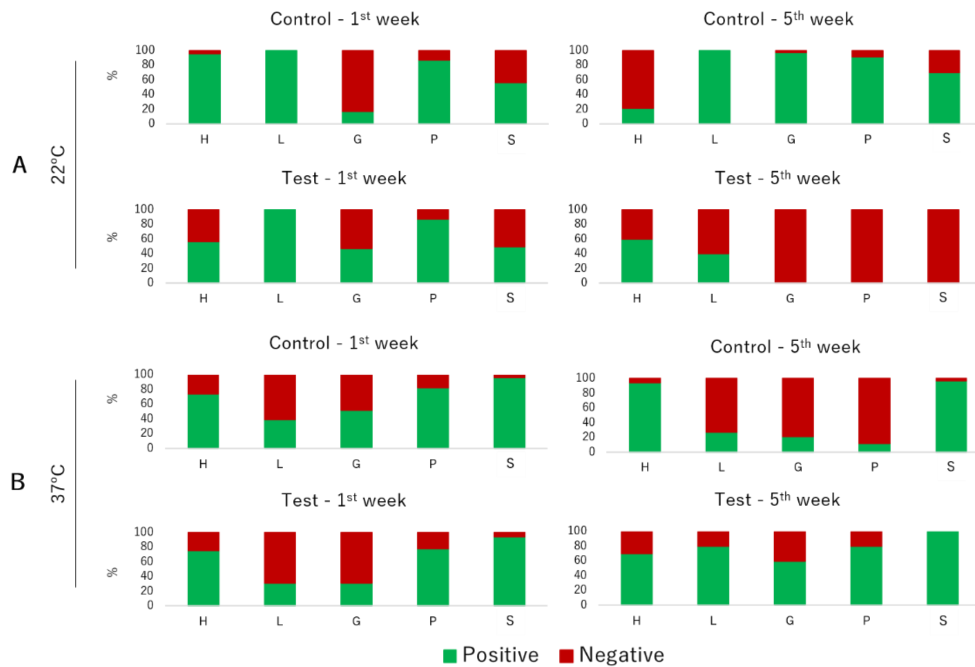


Figure 24. Relative prevalence of virulence factors by tank and sampling week of the isolates analysed at 22°C (A) and 37°C (B). H – haemolytic activity, L – lipase activity, G – gelatinase activity, P – protease activity, S – slime production.

The vast majority of the isolates collected in both tanks were multidrug resistant (79%). Prevalence of multidrug resistant isolates was higher in the control tank (84%) than in the test tank (74.4%). The rate of multidrug resistance varied across the sampling weeks, but all the isolates collected in the fifth week in both tanks were multidrug resistant. The multiple antibiotic resistance (MAR) index values did not differ between experimental conditions ($p=0.911$). However, a variance across the sampling weeks was observed in the fourth week ($p<0.001$) and in the fifth week ($p=0.003$) and MAR index values significantly increased from the first to the fifth week of sampling (Figure 25).

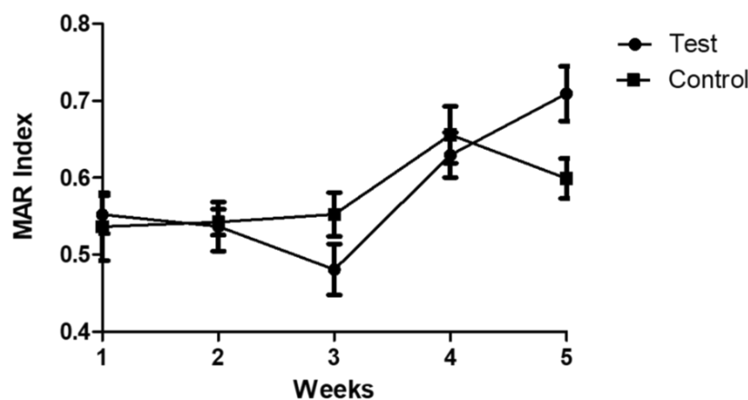


Figure 25. MAR index values (mean + SEM) of the isolates collected in both tanks across the sampling weeks.

The susceptibility dynamics to the tested antimicrobial compounds varied across weeks (Figure 26). Several antibiotics presented significant differences among sampling weeks. For aztreonam, isolates in the first week were more susceptible than those collected in the fifth week of sampling (p=0.038). Similar trends were observed for ceftazidime (p<0.001). Although isolates collected in the third week were more susceptible to enrofloxacin than those from the first week (p=0.017), isolates collected in the fifth week were less susceptible than those from the first week (p<0.001). Regarding imipenem (p=0.026), isolates collected in the fifth week were more susceptible than those collected in the first week. Regarding sulfamethoxazole/trimethoprim, isolates collected in the first week were more susceptible than those collected in the third (p=0.012), the fourth (p=0.004) and the fifth weeks (p=0.016).

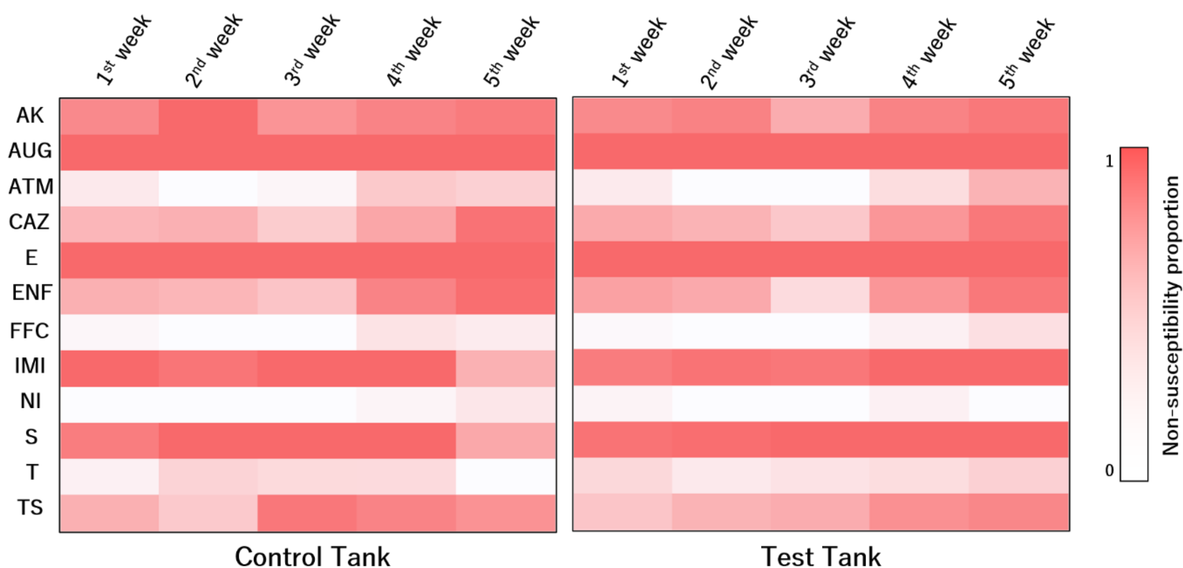


Figure 23. Relative prevalence of non-susceptibility to the tested antimicrobial compounds of the isolates collected in both tanks across the sampling weeks. AK – amikacin, AUG – amoxicillin/clavulanic acid, ATM – aztreonam, CAZ – ceftazidime, E – erythromycin, ENF – enrofloxacin, FFC – florfenicol, IMI – imipenem, NI – nitrofurantoin, S – streptomycin, T – tetracycline, TS – sulfamethoxazole/trimethoprim.

4.5. Discussion

Establishing a successful *ex situ* breeding program is challenging. Along with the difficulties to closely recreate natural habitat conditions for the subject species in the *ex situ* settings, it is fundamental to decrease chances of disease acquisition during the program's duration and to secure limited transmission of important pathogenic agents and their genetic information to natural habitats upon relocation in the wild. Developing husbandry techniques that help mitigate such drawbacks in an *ex situ* program is essential. In this study, we expose,

for the first time, the role of aquatic *ex situ* breeding programs in the amplification of antimicrobial resistance and raise concerns regarding the subsequent introduction of antimicrobial resistance determinants into natural environments upon animal reintroductions into the wild. Similarly, we show that humans working in close association with these programs can acquire important zoonotic bacterial species when safety measures are disregarded. Our results also suggest a trend between the use of protective measures during husbandry of the animals in the *ex situ* program and the decrease of *Aeromonas* spp. prevalence and pathogenic potential.

It is important, however, to acknowledge a major limitation in this study driven by the selected experimental setup. By using only one tank to represent each of the experimental treatments evaluated, and therefore not including replicates that could evidence variability associated with each tank, this study does not fully corroborate the definitive link between the protective measures tested and the observed changes in bacterial dynamics and virulence expression. Although current findings suggest an effect of the tested measures in these parameters, other factors not controlled by the experimental set up could have influenced the dynamics in the tanks differentially. Despite the number of fish individuals available, their intrinsic characteristics and the technical limitations present, the current study is considered an important preliminary investigation in this field and should be complemented with further investigations.

Despite that *Aeromonas* spp. prevalence in fishes was similar among tanks during most of the experimental period, a significant decrease was observed in the last week in the test tank. Similarly, no *Aeromonas* spp. were retrieved from food sources in the test tank, whilst they were detected in the food administered to the animals in the control tank in two of the sampling procedures. It is noteworthy that the isolates collected from the food sources were clones, highlighting a common source of contamination. Members of the genus *Aeromonas* have previously been isolated from fomites (Flores et al. 2021). In a study conducted by Bebak et al. (2015), the risk of *A. hydrophila* outbreaks in cultured catfish significantly increased when animals were seined. Since disinfection protocols, although available (Sadler and Goodwin 2007), are often not regularly implemented, this type of materials represents an important fomite for pathogen introduction into fishes' populations. Current results shed light on the role that materials commonly used during the husbandry of *I. lusitanicum* and other leuciscid species under ex situ conditions, such as hand nets for animal capture and plastic containers for food thawing and preparation, might have in the transmission and persistence of important aquatic pathogens in aquarium tanks used for conservational purposes. Additionally, and since *Aeromonas* spp. are commonly isolated from tap water (Emekdas et al. 2006; Meng et al. 2020), the use of such water to thaw frozen food

for administration can constitute another transmission channel to fishes housed in captive breeding programs.

Although expected, due to the lack of disinfection protocols, it was not possible to isolate *Aeromonas* spp. from the hands and arms of the aquarists involved in this study prior to animal handling. Nevertheless, after handling *I. lusitanicum* individuals, the aquarists' hands and arms yielded *Aeromonas* spp. The zoonotic potential of some species belonging to the *Aeromonas* genus is acknowledged and their role as emergent pathogens has been stressed in recent years (Fernández-Bravo and Figueras 2020). By harbouring a distinct cluster of *Aeromonas* spp., it seems that the most likely route for bacterial acquisition in aquarists was through contact with tank water. Additionally, some isolates collected from the hands of aquarists in distinct weeks were clones, highlighting the success that some strains have in remaining in the tank environment over prolonged periods (Rahman et al. 2007). The lack of biosafety measures such as the use of gloves or hand disinfection results in the acquisition of bacterial pathogens by the aquarists and can evolve into clinical manifestations of disease in certain situations. The development of such infections is possible in people handling aquatic species, being sometimes amplified by cuts and abrasions present in the skin, and can often result in disease ranging from wound infection to sepsis (Lehane and Rawlin 2000; Lowry and Smith 2007). Additionally, this finding highlights the possibility of aquarists acting as vectors of bacterial transmission among animals housed in different settings in captivity. Although further research would be needed to clarify this link, it is important to stress that the use of biosafety measures would likely decrease the probability of pathogen transmission during husbandry actions with aquatic species.

Although *Aeromonas* species structure was similar in the beginning of the trial in both tanks, shifts in the bacterial structure of both tanks were observed across the extent of the experiment. Nevertheless, an increase and predominance of *A. hydrophila* isolates was observed. This similar trend can reflect different explanations. In one hand, it is possible that a similar route of contamination existed for both tanks. As referred before, tap water can be a vehicle for the transmission of *Aeromonas* spp. and the weekly addition of new water to the tanks could have shaped the dynamics observed. Despite we failed to isolate *Aeromonas* spp. in the water samples collected along the experiment, it is possible that this outcome relates to methodological issues rather than lack of *Aeromonas* spp. prevalence in this matrix. On the other hand, and since *Aeromonas* spp. structure in a certain aquatic environment is controlled by its environmental conditions (Pathak et al. 1988; Araujo et. 1991; Rhodes and Kator 1994; Pianetti et al. 2006; Popovic et al. 2015), it is possible that conditions shared by both tanks favoured the development of *A. hydrophila* in detriment of other *Aeromonas* species. Additionally, the species shifts observed for each tank possibly result from the use of protective

measures in the test tank, although a definitive conclusion can't be drawn. Actions that can be met with the introduction of new bacterial strains – i.e., the use of handling material used for individuals living in other tanks and that are not subjected to disinfection protocols – can influence the pre-existing bacterial structure by disrupting the bacterial dynamics originally experienced in the tank. Contrarily, decreasing the external pressures (i.e., by using protective measures) on a determined ecosystem that is in equilibrium will likely retain the original bacterial structure. Another important factor that can't be downplayed are individual fish traits (i.e., genotype, mucus constitution) that can influence bacterial structure in the skin and determine bacterial colonization success. It is also important to acknowledge that, while RAPD analysis may not be considered the most suitable methodology to perform bacterial diversity assessments in *Aeromonas* spp., in the scope of this study, where clone identification and bacterial transmission routes were being evaluated, this tool has proven to be cost-effective and reliable, similarly to previous studies (Szczuka and Kaznowski 2004; Barroco 2013).

A significant reduction of the virulence expression at 22°C was observed in the isolates from the test tank. This possibly occurred due to the absence of activity of selected virulence factors in isolates collected from the test tank. One of the main routes for virulence acquisition in bacteria is through the integration of virulence genes originating from horizontal gene transfer, conferring fitness advantage to the bacterial strain along with an increased pathogenic potential (Beceiro et al. 2013). Contact bridges between different hosts or environments allow the transfer of such genetic information, along with microorganisms carrying genes absent in the original microbiota. In our study, the measures applied in the test tank appeared to prevent the exchange of such virulence determinants between used material and aquarists and *I. lusitanicum* individuals, resulting in a significantly lower virulence potential from the isolates collected in this tank. Another hypothesis is related to the bacterial communities in each tank, that by differing between tanks could also present differential virulence indexes, being then independent of horizontal gene transfer. The same pattern was not observed at 37°C. Temperature is an important driver of virulence gene expression, since bacteria sensor systems perceive environmental modifications and modulate its gene expression (Guijarro et al. 2015; Rasmussen-Ivey et al. 2016a). As a response to heat increase, *Aeromonas* spp. will up-regulate virulence pathways, which will result in the increased production of extracellular products (Guijarro et al. 2015; Pattanayak et al. 2020). As a consequence, *Aeromonas* spp. will display higher virulence and, hence, higher pathogenic potential.

High rates of multidrug resistance levels in the isolates collected in this study are alarming. Additionally, the significant increase of MAR index values in both tanks across the extent of the experiment highlight the role that breeding programs might have in the

dissemination of resistance determinants to wild threatened populations after restocking actions with captive bred animals. Although in this study we focused in aquatic animals, our results are in accordance with previous studies (Power et al. 2013; Stoddard et al. 2009) conducted in *ex situ* conservation programs of terrestrial animals, reinforcing this idea. It seems that the applied protective measures failed to prevent antimicrobial resistance acquisition and dissemination. Since similar prevalence's of non-susceptibility among the isolates from both tanks were registered for the tested antimicrobial compounds, and the observed dynamics across the weeks were equal between tanks, a common source of contamination is the most likely route for the introduction of resistance determinants into the tanks. Despite the fact that different techniques are employed to remove resistance determinants, tap water still presents antibiotic resistance genes (Su et al. 2018). Furthermore, quantity and type of antibiotic resistance genes in the water treatment system are dynamic and dependent on the bacterial species present (Jiao et al. 2018). Hence, it is likely that the acquisition and amplification of antimicrobial resistance in both tanks, as well as the dynamics observed along the sampling weeks, are a reflection of the resistome of the water used in the tanks.

4.6. Conclusion

Biosafety measures should be a critical component of any *ex situ* breeding program, ensuring the successful outcome of the process. In this study, we evaluated the use of protective measures on the prevalence, structure, diversity, virulence expression and antimicrobial susceptibility profiles of a potential fish and human pathogenic genus – *Aeromonas* – and found differences in both prevalence levels and virulence expression in isolates from animals subjected to those measures. Investigations both on the use of biosafety measures, as on antimicrobial resistance and virulence acquisition, lacked in breeding programs of aquatic species. Simple protective measures, such as the disinfection of the handling material and the use of gloves when manipulating animals, can be easily implemented without substantial cost increments and provide significant improvements for the animals' welfare. Although antimicrobial resistance transmission was not prevented with current tested measures, the results in terms of reducing both the prevalence of a potential pathogenic agent, as well as its virulence potential, suggests an advantage in implementing such strategies in aquatic *ex situ* breeding programs. However, further investigations need to be implemented in order to prove this link. Further research should be conducted in order to assess how these strategies affect the animals' microbiome during the captivity time, as a measure of the process disturbance, as well as to investigate efficacy regarding other

important pathogenic agents in fishes. Additionally, methodologies for antibiotic resistance determinants' elimination in drinking water are urged.

Chapter 5. Climatic alterations influence bacterial growth, biofilm production and antimicrobial resistance profiles in *Aeromonas* spp.

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5.1. Abstract

Climate change is expected to create environmental disruptions that will impact a wide array of biota. Projections for freshwater ecosystems include severe alterations with gradients across geographical areas. Life traits in bacteria are modulated by environmental parameters, but there is still uncertainty regarding bacterial responses to changes caused by climatic alterations. In this study, we used a river water microcosm model to evaluate how *Aeromonas* spp., an important pathogenic and zoonotic genus ubiquitary in aquatic ecosystems, responds to environmental variations of temperature and pH as expected by future projections. Namely, we evaluated bacterial growth, biofilm production and antimicrobial resistance profiles of *Aeromonas* species in pure and mixed cultures. Biofilm production was significantly influenced by temperature and culture, while temperature and pH affected bacterial growth. Reversion of antimicrobial susceptibility status occurred in the majority of strains and tested antimicrobial compounds, with several combinations of temperature and pH contributing to this effect. Current results highlight the consequences that bacterial genus such as *Aeromonas* will experience with climatic alterations, specifically how their proliferation and virulence and phenotypic resistance expression will be modulated. Such information is fundamental to predict and prevent future outbreaks and deleterious effects that these bacterial species might have in human and animal populations.

5.2. Introduction

Environmental conditions are a major driver of bacterial activity and can shape the expression of several metabolic pathways (Parter et al. 2007; Ratzke and Gore 2018). Namely, such parameters have the potential to influence bacterial virulence (e.g., biofilm formation) and antibiotic resistance signatures (De Silva et al. 2018; Huang et al. 2018).

Climatic scenarios, as predicted by simulation methodologies based on different levels of emissions, are projected to significantly differ from currently observed meteorological conditions (Pachauri et al. 2014). Regarding aquatic ecosystems, and particularly in freshwater habitats, various environmental parameters are expected to be altered in the coming years. Water temperature, directly influenced by air temperature, is expected to rise across different habitats (Knouft e Ficklin 2017). Additionally, the occurrence of heatwaves will likely increase, resulting in extended periods of drought associated with a low flow of freshwater systems, a decrease in water level and in dissolved oxygen concentrations (Van Vliet et al. 2011; Bucak et al. 2017; Griffith and Gobler 2020). Consequently, reduced dilution of freshwater streams will also affect ion balance levels (Kaushal et al. 2018; Le et al. 2019). These biotic changes will impact ecosystem dynamics and promote disruptions in species equilibrium (Griffith e Gobler 2020; Dudgeon 2019). All of these events are expected to

significantly decrease freshwater's quality (Mosley 2015). Ultimately, these changes compromise future water availability, freshwater ecosystems' structure and populations' sustainability (Van Vliet et al. 2013; Pinceel et al. 2018; Rodell et al. 2018).

Natural aquatic ecosystems, often the last destination of terrestrial runoffs, are known reservoirs of both antimicrobial resistance and bacterial virulence determinants (Chen et al. 2018). The microbiota present there, with or without direct connection with clinical infections, constitute a pool of information to the terrestrial microbiota or can even be disseminated to anthropogenic cycles (Peterson et al. 2018). This intricate connection between environmental microbiota and bacterial genus with effects at the One Health level stresses the importance of close surveillance of antimicrobial resistance and virulence dynamics in natural habitats in order to prevent epidemic situations both in anthropogenic settings and natural habitats (Alexander et al. 2015; Dias et al. 2020). Since modelling bacterial responses to changing environmental parameters in natural habitats is challenging, lab simulations—e.g., microcosm assays—are an important tool to predict how microbiota will respond to environmental cues foreseen in climatic predictions (Friman et al. 2011; Saarinen et al. 2019).

We hypothesize that aquatic bacteria's antimicrobial resistance signatures and virulence traits, as well as their growth, may vary with changing environmental conditions. In order to test this, we applied microcosm simulation assays using different water temperatures and pH values following established emissions scenarios (Pachauri et al. 2014) to *Aeromonas* spp.—a model bacterial genus ubiquitous across different aquatic ecosystems—and evaluated changes in the antimicrobial resistance profile, biofilm production and growth of the isolates under study.

5.3. Materials and Methods

5.3.1. Strain selection

Aeromonas species selection followed results obtained prior to this study (Grilo et al. 2021). Namely, the occurrence of mesophilic *Aeromonas* spp. was investigated in *Iberochondrostoma lusitanicum* in four freshwater streams in the Lisbon district, Portugal (Lizandro: 38.886701°, −9.298140°; Samarra: 38.894761°, −9.433734°; Jamor: 38.720832°, −9.249696°; Laje: 38.709159°, −9.314079°) previously characterized by our team (Sousa-Santos et al. 2016). *A. caviae*, *A. hydrophila*, *A. media* and *A. veronii* were considered the most abundant species and, hence, included in this study. Strains were selected from a bacterial library evaluated by a RAPD (random amplified polymorphic DNA) technique in order to perform molecular typing and genomic differentiation. Three isolates of each *Aeromonas*

species that were not considered clones, originating from different locations, were selected as representatives for inclusion in the study (*n* = 12).

The strains' ability to produce slime was evaluated using a phenotypical assay, Congo Red Agar (22 °C, 72 h), as described before (Freeman et al. 1989). Only slime-producer strains were selected for inclusion in the study.

Strains were stored in pure cultures in cryovials stored at −80 °C. Prior to their use, resuscitation was performed by transferring 100 µL of each bacterial suspension to 8 mL of Brain Heart Infusion broth (BHIB; VWR, Radnor, PA, USA), incubating for 24 h at 21 °C. After, bacterial suspensions were transferred to solid mediums—BHI agar and Columbia Blood (COS) agar (Biomérieux, Marcy-l'Étoile, France)—and incubated at 21 °C for 24 h. The purity of the cultures was confirmed by macro and microscopic morphology, as well as by Gram staining and phenotypic traits (oxidase production).

5.3.2. Biofilm formation quantification

In order to standardize the number of colony-forming units (CFU) in the suspensions to be used in the quantification of biofilm formation, reference *Aeromonas* strains were selected, namely *A. caviae* ATCC 1976, *A. hydrophila* ATCC 7966, *A. media* ATCC 33907 and *A. veronii* ATCC 35624.

Briefly, reference strains were incubated in BHI agar and COS agar at 21 °C for 24 h. After incubation, for each reference strain, colonies were selected and inoculated in 5 mL of 0.9% saline solution until adjusting to a turbidity of 0.5 McFarland using a digital densitometer DENSIMAT (Biomérieux, Marcy-l'Étoile, France). After homogenization, serial ten-fold dilutions were performed in 9 mL of 0.9% saline solution (up to 10^{-6}). From each dilution (10^{-4} to 10^{-6}), 100 µL were collected and plated in BHI agar in duplicate, using sterilized glass beads. Plates were incubated at 21 °C up to 48 h. Colonies were counted in both plates and averaged. The number of CFU/mL was calculated using the formula (number of colonies × dilution factor)/volume.

Biofilm formation was performed using the microtiter plate assay and quantification was performed using the crystal violet method, as described before (Stepanović et al. 2000, 2007) with modifications. Bacterial colonies were collected from BHI agar and suspended in 5 mL of 0.9% saline solution until adjusting to a turbidity of 0.5 McFarland. Based on the pre-established average CFU/mL for each *Aeromonas* species, concentrations were adjusted for each strain in order to prepare a final concentration in the wells of the Nunc™ MicroWell™ 96-well plates (ThermoFisher Scientific®, Waltham, MA, USA) of 5×10^5 CFU/mL in a final volume of 200 µL. As culture medium, Tryptic Soy Broth (TSB, VWR, Radnor, PA, USA)

supplemented with 0.25% glucose (Millipore®, Merck, Darmstadt, Germany) was used. *A. hydrophila* ATCC 7966 is considered a strong biofilm producer; hence it was selected as a positive control. As a negative control, TSB supplemented with 0.25% glucose was used in six wells in each assay. The microtiter plate was incubated at 21 °C for 48 h.

After incubation, the content of all wells was carefully aspirated to eliminate planktonic forms and the wells were washed three times at room temperature with phosphate-buffered saline (PBS; VWR, Radnor, PA, USA) at pH 7.0. The PBS was discarded after the final wash and the microtiter plate was incubated in an inverted position at 60 ˚C for 1h, for the adherent cells to fixate. After, 150 µL of 0.25% Hucker crystal violet (diluted in de-ionized water; Merck, Darmstadt, Germany) were added to the wells, followed by incubation at room temperature for 5 min. The stain excess was aspirated, and the microtiter plate rinsed until the rinse was free of stain. The microtiter plate was airdried at room temperature and, once dry, 150 µL of 95% ethanol (NORMAPUR®, VWR, Radnor, PA, USA) were added to each well for solubilization of the stain. The microtiter plate was covered with the lid to avoid ethanol's evaporation and incubated at room temperature for 30 min. After incubation, the optical density (OD) of the microtiter plate was evaluated at 570 nm in a horizontal bidirectional reading using the FLUOstar OPTIMA microplate reader (BMG LABTECH, Ortenberg, Germany). This assay was performed prior and after the microcosm assay to enable further comparisons. In both situations, three replicates were performed for each strain on independent days.

5.3.3. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the disk diffusion technique (Bauer et al. 1966). Guidelines of the Clinical and Laboratory Standards Institute for *Aeromonas salmonicida* testing were followed as reference (CLSI 2014), selected since the testing temperature—22 °C—closely resembles the temperature used for the basal treatment. The following antibiotics (Mastdiscs®, Mast Group, Liverpool, UK) were tested: erythromycin (E, 15 µg), tetracycline (T, 30 µg) and sulfamethoxazole/trimethoprim (TS, 23.75–1.25 µg). Antimicrobial compound choice followed options where epidemiological cut-off values were available. A "wild-type" (WT) phenotype implies isolate susceptibility to the antimicrobial, while a "non-wild-type" (NWT) phenotype implies that the isolate presents resistance mechanisms. *Escherichia coli* ATCC 25922 was used as a quality control. This technique was performed prior and after the microcosm assay to enable further comparisons. One strain from each species was randomly selected to be tested. Only strains from pure culture microcosms (i.e., no strains from mixed cultures microcosms were used) were used to perform the antimicrobial susceptibility testing. The same strain was used prior and after microcosm comparisons. In both situations, 10% of replicates were performed on independent days.

5.3.4. Microcosm assay

To evaluate the influence that water temperature and pH might have in the antimicrobial resistance and virulence profiles of *Aeromonas* spp., a microcosm simulation assay was developed. Testing variables (i.e., temperature and pH) were selected based on the expected impact that climatic alterations will have in these two parameters in freshwater ecosystems (Pachauri et al. 2014) and on the known influence of these variables on bacterial biofilm formation and resistance acquisition/expression (Goller and Romeo 2008; Toyofuku et al. 2016; Cruz-Loya et al. 2018; MacFadden et al. 2018; Mueller et al. 2019).

Regarding water temperature, four experimental conditions were used. First, a condition representing the current water temperature values was created based on trends in water temperature observed during higher temperature months (July to October) in the Lisbon's District rivers (Cascais, Oeiras and Sintra municipalities) in the period between 1985–2016 and averaged (21 °C) (SNIRH 2021). Only sampling points located far from the river mouth were selected to prevent temperature oscillations related to other water bodies. Similarly, only sampling points with substantial datasets over a wide temporal frame were selected (n = 6). Location was selected to match the origin of the bacterial isolates. Additionally, two different 21st-century projections of climate alterations for the period of 2081–2100 establishing different levels of greenhouse gas emissions and atmospheric conditions, air pollutant emissions and land use were selected—representative concentration pathways (RCP) 4.5, representing a scenario of medium stabilization (23.2 °C) and 8.5, representing a scenario of high warming (24.5 °C) (Pachauri et al. 2014). To mimic a scenario of rapid temperature fluctuations, the protocol established by Saarinen et al. (2019) was implemented with modifications to accommodate *Aeromonas* spp. growth conditions and the temperature ranges defined for this study. So, repetitions of 24 h cycles of either 24.5 °C or 21 °C were applied. Additionally, to establish an initial time point to enable comparisons in both the microtiter plate assay and the disk diffusion technique prior and after the microcosm assays, a treatment (T0) mimicking the current water temperature and pH (21 °C, pH 7.61) was included. Contrarily to the other treatments, the strains in T0 were incubated in river water for only 24 h.

Simulations from van Vliet et al. (2011) on the correlation between air and river water temperature were used to determine final water temperature conditions for the RCP scenarios. Additionally, river discharge level, which also affects water temperature, was based on simulations by van Vliet et al. (2011, 2013) for the Iberian Peninsula and fixed at decrease levels of 40%.

Regarding water pH, and since this parameter trends in rivers will vary according to demographic and geologic characteristics of the areas adjacent to the river (Kaushal et al.

2013; Oberholster et al. 2017), both a scenario of acidification and a scenario of alkalization were included. Three conditions were created, two mimicking both previously described scenarios and one establishing the current water pH conditions. Water pH values were established based on trends accessed in the same datasets used for temperature (SNIRH 2021). The treatment established as the current condition was based on the average of the values recorded in the analysed period (pH 7.61). The acidification scenario was based on the average of the lowest pH values observed in all analysed rivers (pH 6.31), while the alkalization scenario was based on the average of the highest pH values recorded (pH 8.61). A summary of the experimental conditions used in this study is found in Table 7.

Table 7. Experimental conditions used in the microcosm assays. RCP—representative concentration pathway.

Experimental Conditions			
Temperature (°C)		pH	
Current	21	Current	7.61
RCP 4.5	23.2	Acidification	6.31
RCP 8.5	24.5	Alkalization	8.61
Fluctuations	21–24.5		

Microcosm experimental setup was adapted from Zhang e Buckling (2012) and Cairns et al. (2017). Water preparation was performed as described in Sautour et al. (2003). BHIB was used as an addictive of river's water to act as a nutrient source. This medium was used at a 2.5% concentration to resemble the resource levels found in natural ecosystems.

Briefly, river water collected in a freshwater stream in the Lisbon district (Jamor: 38.720832°, −9.249696°) was filtered using a 0.22 μm Millipore filter (Frilabo, Maia, Portugal) and autoclaved at 121 °C for 20 min. For each water pH condition, BHIB was added to the water and pH adjusted to match the conditions established using a HI-4521 Research Grade pH/ORP/EC Bench Meter (Hanna Instruments, Póvoa de Varzim, Portugal). Bacterial suspensions were prepared by collecting colonies from BHI agar that were suspended in 5 mL of 0.9% saline solution until achieving a turbidity of 0.5 McFarland. Suspensions were prepared in pure cultures and in mixed cultures (with only one strain of each species—*A. caviae*, *A. hydrophila*, *A. media* and *A. veronii*—represented once). Nunc™ MicroWell™ 96-well plates were used to establish the microcosm. In pure culture wells, 200 μL of the respective medium were added, following the addition of 10 μL of the bacterial suspension. In the mixed culture wells, 2.5 μL of each bacterial strain was used. In both situations, bacterial suspensions were prepared in 0.9% saline solution previously according to the established average CFU/mL of the reference strains to achieve a final concentration of 5×10^5 CFU/mL

in each well. In the negative control wells, 210 µL of the respective medium was added. Plates were incubated for 6 days in the respective temperature treatment inside an SSI10 SSI10-2 orbital shaking incubator (Shel Lab, Cornelius, NC, USA) at 150 rpm to mimic water turbulence in the natural habitat. Every 48 h of incubation, renewal of the medium was performed by adding 20 µL of the previous culture into a new plate with 180 µL of the respective medium. At the end of each microcosm assay, the OD was read at 570 nm as described before to determine bacterial growth. After reading, 10 µL from each well was transferred into BHI agar, incubated at the respective assay's temperature for 24 h and used for biofilm quantification, antimicrobial susceptibility testing and species confirmation (in the case of the mixed culture wells). The pH values for each assay were validated by randomly selecting bacterial cultures across the three different pH used, as well as the negative controls mediums, and analyzed using Neutralit® pH-indicator paper (Merck, Darmstadt, Germany). Tests were performed immediately after incubation.

5.3.5. *Aeromonas* species confirmation in mixed culture wells

Following the microcosm assays, species confirmation in the mixed culture wells was performed. Bacterial colonies with distinct macroscopic morphology in BHI agar were selected and streaked into pure cultures. The purity of the cultures was evaluated by macro and microscopic analysis, and Gram staining and oxidase production were evaluated.

Bacterial genomic DNA was obtained by the boiling method (Talon et al. 1998). To achieve species identification, a multiplex PCR protocol previously described (Persson et al. 2014) was used with some modifications. This protocol targets the identification of the four species included in this study. *A. caviae* ATCC 1976, *A. hydrophila* ATCC 7966, *A. media* ATCC 33907 and *A. veronii* ATCC 35624 were used as positive controls.

Briefly, PCR mixtures were performed in a final volume of 25 µL, composed of: 12.15 µL of Supreme NZYTaq 2 × Green Master Mix (NZYTech, Lisbon, Portugal), 10 µL of PCR-grade water (Sigma-Aldrich, Saint Louis, MO, USA), 0.025 µL (0.05 µM) of primers A-16s, 0.25 µL (0.5 µM) of primers A-cav, 0.1 µL (0.2 µM) of primers A-med, 0.225 µL (0.45 µM) of primers A-hyd, 0.075 µL (0.15 µM) of primers A-Ver; and 1.5 µL of template DNA. Thermocycler conditions included a hot start at 95 ◦C for 2 min; followed by 6 cycles of denaturation at 94 ◦C for 40 s, annealing at 68 ◦C for 50 s and extension at 72 ◦C for 40 s; and 30 cycles at 94 ◦C for 40 s, 66 ◦C for 50 s and 72 ◦C for 40 s.

Amplification products were resolved by gel electrophoresis using 1.5% (*w*/*v*) agarose in 1 × TBE Buffer (NZYTech, Lisbon, Portugal). Gels were resolved for 45 min at 90 V and NZYDNA Ladder VI (NZYTech, Lisbon, Portugal) was used as a molecular weight marker.

Gels were visualized using a UV light transilluminator. The images were recorded through the Bio-Rad ChemiDoc XRS imaging system (Bio-Rad Laboratories, Hercules, CA, USA).

5.3.6. Data & Statistical analysis

Prior to statistical analysis, the influence of the microcosm assay (i.e., other factors than the water temperature and pH conditions) on the biofilm production and antimicrobial resistance profiles were accessed by comparing the results obtained with the treatment T0 and current pH 7.61 (similar water temperature and pH conditions). A coefficient of variation of 25% was set as a breakpoint and calculated individually for each *Aeromonas* species. Minimal and maximal limits were calculated regarding T0 values. Current pH 7.61 values that fell outside the limit were considered significantly different. Replicates of isolates where this situation occurred were excluded from the subsequent analysis due to possible bias (i.e., *A. veronii* #1 3rd replicate, mixed culture #1 2nd and 3rd replicates, mixed culture #2 2nd replicate, mixed culture #3 1st and 2nd replicates). For antimicrobial resistance profiles, a qualitative comparison of the epidemiological cut-off values between the two treatments were performed and no deviations occurred.

Several isolate level response variables were analysed regarding temperature and pH treatments. Using a factorial ANOVA where it was determined the difference in values regarding T0 treatment and Tukey's multiple comparison test to evaluate differences between treatments, the (1) biofilm production and the (2) bacterial growth were considered. Using a stepwise linear regression and a point-biserial correlation, the influence of the different *Aeromonas* species in mixed cultures on the production of biofilm was considered. Pearson's correlation was calculated between biofilm production and bacterial growth. The statistical analysis was performed using IBM SPSS Statistics version 27 software (IBM Analytics, New York, NY, USA). Graphs were produced using GraphPad Prism® (GraphPad Software, San Diego, CA, USA, version 5.01).

5.4. Results

Biofilm production by each of the *Aeromonas* strains in pure and mixed culture in the different assays is illustrated in Figure 27. Each strain's response to temperature and pH was variable between species and within the same species.

When considering results by groups (*Aeromonas* species individually and mixed cultures), biofilm production in the mixed cultures' wells was significantly lower ($p<0.001$) than in the other groups. Additionally, water temperature also significantly influenced biofilm production ($p=0.006$), with isolates exposed to the Fluctuations treatment producing less biofilm (Figure 28). The different pH conditions tested did not influence biofilm production.

Regarding mixed culture wells, re-isolation and identification of the initial *Aeromonas* pool added to each well was not possible with several combinations of temperature and pH treatments. *Aeromonas* species prevalence at the end of microcosm assays varied across the applied treatments and also between replicates (Figure 29). When evaluating the influence of each individual *Aeromonas* species present in mixed cultures on the biofilm production, it was observed that no species had a significantly different influence.

Some differences were observed regarding the growth of the isolates during the experiment (Figure 30). Significant differences were recorded between the tested *Aeromonas* species ($p<0.001$). *A. veronii* isolates presented significantly lower concentrations than the other single and mixed cultures, while *A. hydrophila* presented significantly lower concentrations than *A. media* and mixed cultures. Temperature ($p<0.001$) and pH ($p=0.007$) treatments also influenced bacterial growth. While bacterial growth did not differ between current and fluctuations treatments, it was significantly increased in the RCP 4.5 treatment and decreased in the RCP 8.5 treatment. Bacterial growth was increased in acidic pH conditions (6.31) when compared to alkaline pH (8.61). Specific associations were also found between *Aeromonas* species and pH ($p=0.002$) and between temperature and pH ($p<0.001$). While *A. media* and mixed cultures presented higher concentrations in water microcosms with pH 6.31, *A. caviae* presented higher concentrations at pH 8.61. No differences were observed at pH 7.61. Regarding the interaction between temperature and pH, concentrations in the RCP 4.5 treatment were higher at pH 6.31, decreasing until pH 8.61. For RCP 8.5, higher concentrations were observed at pH 8.61.

The bacterial concentration was not correlated with biofilm production ($r_s=0.020$, $p=0.676$).

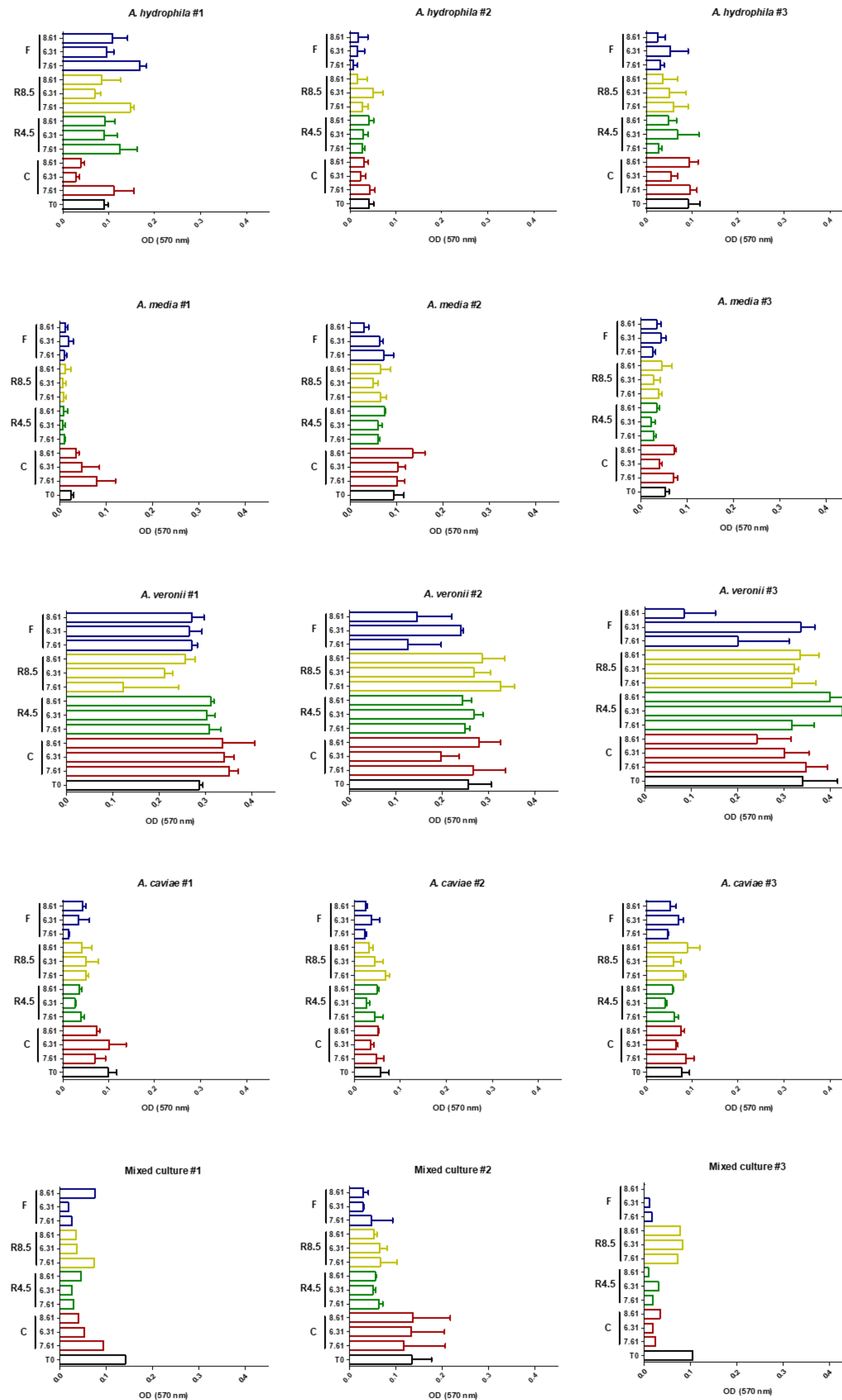

Figure 247. Biofilm production by each strain and mixed culture (mean + SEM). Presented results correspond to values subtracted to each treatment's negative control for normalization. The three replicates' results are presented by strain and mixed cultures, except for replicates where T0 and Current pH 7.61 were considered significantly different (*A. veronii* #1, Mixed cultures #1, #2 and #3). First column in each graph represents the temperature treatment (C—Current, R4.5—RCP 4.5, R8.5—RCP 8.5, F—Fluctuations) and the second the pH treatment. OD—Optical density.

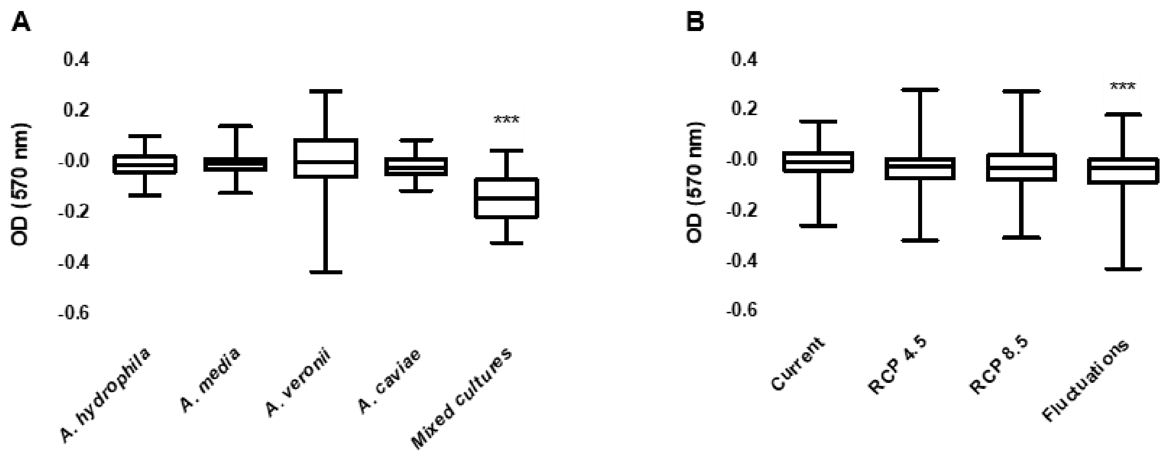

Figure 25. Biofilm production by individual *Aeromonas* species and mixed cultures (A) and in different water temperature treatment – each treatment includes all strains results. (B). Presented results correspond to values subtracted to each treatment's negative control for normalization and to the corresponding T0 treatment values for comparison. OD—Optical density; *** p<0.001.

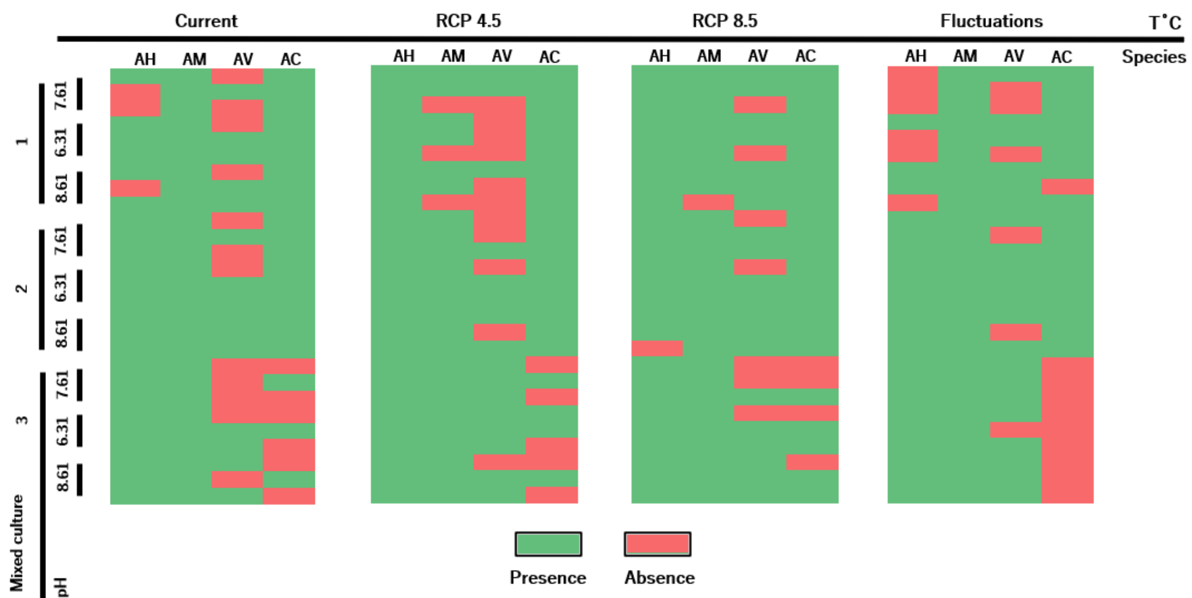

Figure 26. Prevalence of *Aeromonas* species in the mixed cultured wells after the microcosm assay. Each line corresponds to a distinct replicate belonging to one of the pH treatments (7.61, 6.31 and 8.61) from the tested mixed cultures (#1, #2 and #3). Each column represents an *Aeromonas* species (AH—*A. hydrophila*, AM—*A. media*, AV—*A. veronii*, AC—*A. caviae*) from a specific temperature treatment (Current, RCP 4.5, RCP 8.5 and Fluctuations).

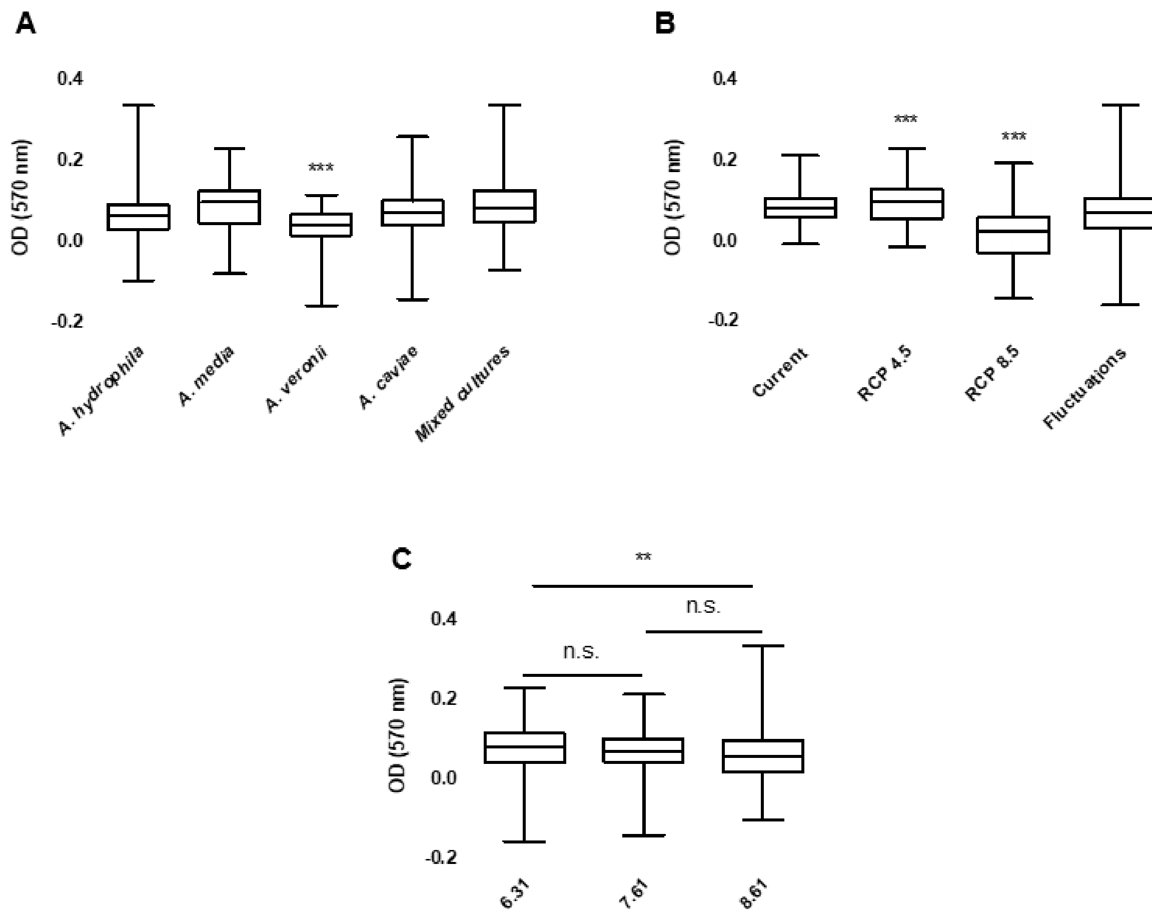

Figure 270. Bacterial concentration by *Aeromonas* species and mixed cultures (A), by water temperature treatment (B) and by water pH treatments (C). Presented results correspond to values subtracted to each treatment's negative control for normalization and to the corresponding T0 treatment values for comparison. OD—Optical density. n.s. $p>0.05$, ** $p<0.01$, *** $p<0.001$.

Several changes regarding the antimicrobial resistance profile were observed among treatments for the same isolate (Figure 31). Observations between the control treatment (T0, pH 7.61) were similar to results obtained with the current treatment and similar pH levels. Phenotype variation occurred in a strain-dependent way, and it was specific for each antimicrobial compound tested. For all strains and antibiotics (except *A. hydrophila* and tetracycline), modification of the original susceptibility category occurred with at least one combination of treatments.

In certain situations, reversion of non-wild-type to a wild-type phenotype occurred only with specific combinations of temperature and pH. This is the case of erythromycin susceptibility and *A. caviae*, *A. hydrophila* and *A. media*. Regarding *A. caviae* and *A. media*, the same treatment (i.e., Current and pH 6.31) caused this phenomenon. In other cases, several combinations resulted in this reversion with no obvious pattern. The opposite was also

observed (conversion from wild-type to non-wild-type) among the isolates. Although some treatments seemed to result in this situation more often for some antimicrobial compounds (i.e., RCP 4.5), a high variability was observed.

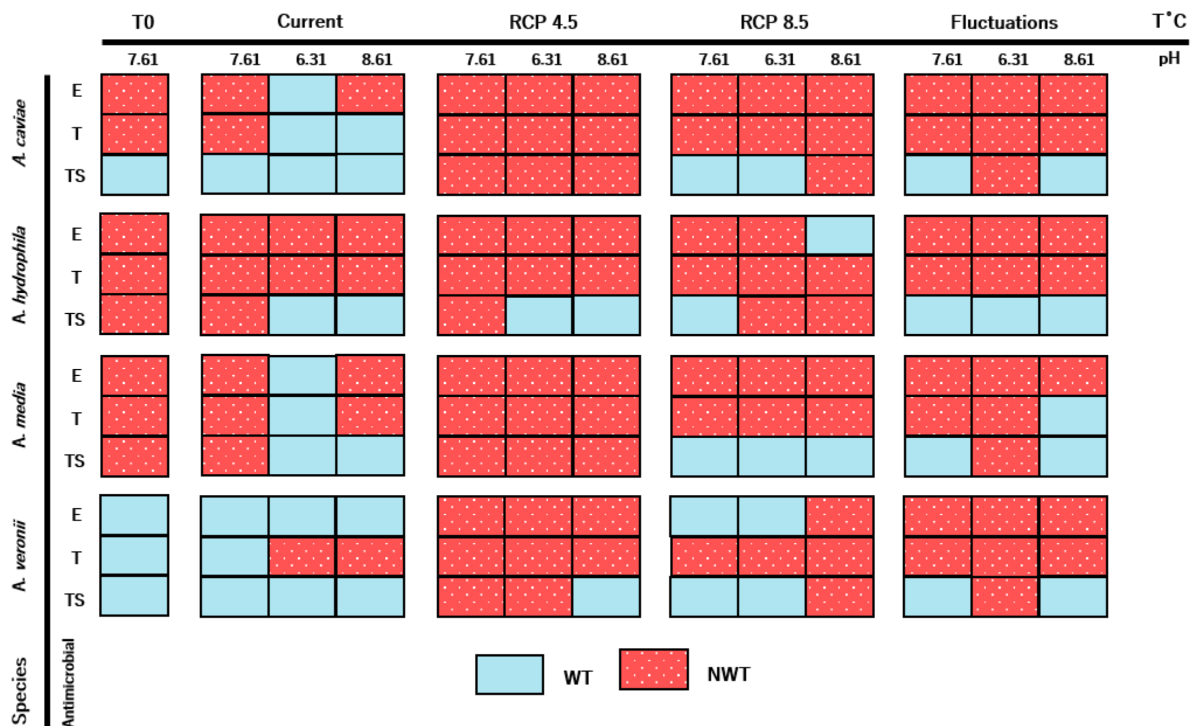

Figure 281. Antimicrobial resistance phenotypes (WT—wild-type, susceptible; NWT—non-wild-type, non-susceptible) of the *Aeromonas* isolates regarding water temperature and pH treatments. E—erythromycin, T—tetracycline, TS—sulfamethoxazole/trimethoprim.

5.5. Discussion

Investigating how bacteria will evolve with environmental cues using natural habitats is a difficult task. Instead, the use of microcosm simulations allows the exploration of such associations, ensuring experimental control and uniformity. This methodology represents a first step in the prediction of transformations to occur in important bacterial genus with an impact at the One Health level, such as *Aeromonas* spp., and prepare for future outbreaks or phenotypical changes with consequences to public health. In this study, we show that different *Aeromonas* species adapt their growth, biofilm production and antimicrobial resistance signatures to environmental projections related to climatic alterations (i.e., temperature and pH) in water, highlighting the role that future climatic events will have in shaping bacterial activity, as well as virulence and resistance expression. It is noteworthy that, in this study, differences regarding growth, biofilm production and antimicrobial resistance signatures were

observed using relatively small temperature and pH amplitudes, which are more likely to reflect future climatic trends.

5.5.1. Biofilm production

In general, the studied isolates presented variability in the production of biofilm when exposed to the different temperature and pH treatments. Although some response patterns were present, the disparity in results between isolates of different species and within the same species highlights the fact that individual characteristics will govern how an isolate will respond to environmental cues; however, significant associations were observed. Mixed cultures produced significantly less biofilm when compared to the *Aeromonas* species individually. At the end of the microcosm assay, it was not possible to isolate all *Aeromonas* species in many mixed cultures. Some species absence was more evident than others (e.g., *A. caviae* in mixed culture #3 along the various temperature and pH treatments), although a general pattern was not present. Additionally, and while pH treatments seem not to influence biofilm production significantly, temperature influenced biofilm production in *Aeromonas* spp. Namely, isolates exposed to temperature oscillations (i.e., Fluctuations) produced less biofilm. Such biofilm production was not dependent on bacterial concentration. Distinct *Aeromonas* species display specific preferences regarding environmental parameters (Abbott et al. 2003; Wang and Gu 2005). Although *Aeromonas* spp. possess stress response mechanisms to deal with environmental oscillations (Awan et al. 2018), they still impact several aspects of bacterial life. If the combined temperature and pH conditions fall within the optimal range for multiplication and virulence expression for each isolate, they will dictate the isolate's competitiveness and ability to survive in an environment composed of multiple species (Thomas et al. 1996; Lopez-Vazquez et al. 2009). Further, the level of interspecific competition for the limited resources will also hinder each isolate's ability to allocate nutrients to processes such as biofilm production, contrary to what occurs in pure cultures (Yuan et al. 2020). Finally, environmental oscillations of abiotic factors, such as temperature, will create additional disturbances for the bacterial communities (Saarinen et al. 2019) and the overall combination of external stressors with internal competition is likely to impact the final biofilm production.

5.5.2. Bacterial growth

In this study, a disparity in bacterial growth during the microcosm experiments was observed between the studied species (both in pure culture and in mixed culture). Overall, *A. veronii* isolates displayed a significantly lower growth when compared with other tested groups. Delamare et al. (2000) highlighted lower growth patterns by *A. veronii* when compared to other *Aeromonas* species (i.e., *A. hydrophila*, *A. media* and *A. caviae*). Growth rate

variability is a consequence of phenotypic diversity in bacteria (Nana et al. 2018). Such variability can be the result of the nutrient uptake rate by the bacterial cell and of the resource's distribution between the processes occurring in the bacterial cell (Goelzer and Fromion 2011). *Aeromonas* strains and species growth variability likely reflect different limitations in these processes among the isolates, which can also explain differences observed not only for *A. veronii* but also for *A. hydrophila*. In the mixed cultures group, this pattern was not observed, and two hypotheses can be drawn: either other *Aeromonas* species present in the culture compensated for lower growth rates by *A. veronii*, or interspecific competition eliminated *A. veronii* presence in the microcosm wells (as stated in Figure 3), facilitating the growth of other species or cancelling the growth effect *A. veronii* had in the total growth.

Both changes in water temperature, as well as in the pH conditions, played a significant role in the growth of *Aeromonas* spp. Regarding temperature, a biphasic effect was observed: while small increments in water temperature (i.e., RCP 4.5) seem to benefit the *Aeromonas* species under study, both in pure and in mixed cultures and favour their proliferation; once reaching a certain threshold imposed by higher temperatures (i.e., RCP 8.5), such boosting effect is lost and bacterial growth is lowered. Temperature is a determinant in bacterial growth and *Aeromonas* typically increase both growth and metabolic activity and decrease lag phase when experiencing higher environmental temperatures (Cavari et al. 1981; Sautour et al. 2003); however, such growth reaches a plateau with temperature increments and starts to decrease before reaching maximum thermal tolerance (Palumbo et al. 1985), highlighting the role of thermal stress as a regulator of bacterial growth. It is noteworthy that, although cultures subjected to the fluctuation treatment experienced similar temperature values, such as the ones in the RCP 8.5 treatment, alternate exposure to higher (24.5 °C) and lower (21 °C) temperature values likely created buffer periods in which bacterial cultures could stabilize and multiplicate.

Regarding pH, the overall growth of *Aeromonas* spp. was higher in acidic environments when compared to alkaline environments. While some authors found a non-significant effect or a negative effect of pH on *Aeromonas* growth (Knøchel 1990; Sautour et al. 2003; Vivekanandhan et al. 2003), *Aeromonas* are evolutionarily adapted to low pH environments, such as the gastrointestinal environment, and have built cellular responses (i.e., protective protein synthesis) that allow for acid tolerance (Karem et al. 1994). Additionally, when exposed to acidic environments, the lag phase in *Aeromonas* is significantly shorter, prompting the beginning of the following growth phases sooner (Buncic and Avery 1995); however, it is likely that different *Aeromonas* species display specific niche preferences and have evolved towards tolerance in different pH gradients. This explains why in this study some groups exhibited higher growths in acidic treatments (*A. media* and mixed cultures), while others performed

better in alkaline pH (*A. caviae*). Additionally, both temperature and pH seem to play an interactive role, conditioning higher growth of *Aeromonas* spp. with specific combinations (i.e., RCP 4.5 and acidic pH, RCP 8.5 and alkaline pH).

5.5.3. Antimicrobial resistance profiles

Climate change has been implicated as a factor involved in increasing levels of antimicrobial resistance among different bacterial species in prolonged temporal sets. Distinct spatial patterns occur globally and are connected with local climacteric variability, highlighting how distinct geographical areas will be impacted by this problem in different proportions (Alvarez-Uria and Midde 2018; Kaba et al. 2020). Specifically, regions expected to be more vulnerable to climacteric alterations are also the ones predicted to accumulate the highest prevalence of antimicrobial resistance (Reverter et al. 2020). Some authors report the role of increasing temperatures over time in the overexpression of this phenomenon in species such as *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (MacFadden et al. 2018; Kaba et al. 2020). In a meta-analysis with isolates collected in aquacultures conducted by Reverter et al. (2020), a similar conclusion was drawn for bacterial genera commonly infecting aquatic animals. In this study, we show that climatic scenarios of changing temperature and pH can alter the antimicrobial susceptibility profile of different *Aeromonas* species. Although species belonging to the *Aeromonas* genus are normally resistant to erythromycin and susceptible to tetracycline and sulfamethoxazole/trimethoprim, the selected strains in this study displayed variable susceptibility status to these antimicrobials; however, and with the exception of one strain (*A. hydrophila* and tetracycline), reversion of the original susceptibility status occurred for all tested strains and antimicrobial compounds at least in one experimental condition.

In some situations, reversion of non-susceptibility to susceptibility to the tested antimicrobial compounds was observed. Antibiotic resistance represents a fitness cost for bacterial species and the development of resistance is modulated by this parameter (Anderson and Hughes 2010; Sundqvist 2014). Resistance to antimicrobial compounds can impact important cellular activities or be met with higher energetic costs related to gene expression needs (Melnyk et al. 2015; Hernando-Amado et al. 2017). Thus, when experiencing amplified fitness costs, such as those provided by changes in temperature and pH, the rate of resistance reversibility in bacteria increases (Andersson and Hughes 2010). In this study, it seems that several combinations of water temperature and pH treatments resulted in the phenomenon that accommodates this hypothesis; however, resistance development was also observed in this study for strains displaying wild-type status. In alternative to resistance acquisition through horizontal gene transfer, a process known to be modulated by temperature conditions

(MacFadden et al. 2018), *de novo* mutations (including recombination) can explain antibiotic resistance development in the absence of resistance determinants or antimicrobial pressure in the environment (Melnyk et al. 2015; Hernando-Amado et al. 2017), as in this study. In fact, increasing temperatures have been associated with genome-wide selection of these mutations (Berger et al. 2021). Despite the costs in fitness already described for resistance acquisition, bacterial species have the potential to downplay such costs by means of compensatory evolution by developing mutations that will decrease fitness cost without compromising antimicrobial resistance or by performing physiological adaptations or activating specific systems that buffer mutational effects and fitness costs (Melnyk et al. 2015; Freihofer et al. 2016; Hernando-Amado et al. 2017; Fay et al. 2021). Different factors can influence the acquisition of antibiotic resistance in these settings, such as thermal stress or changes in pH (McMahon et al. 2007; Rodríguez-Verdugo et al. 2013). Antimicrobial resistance development occurred in this study for several combinations of water temperature and pH treatments. It is likely that the final antimicrobial susceptibility of the isolates corresponds to an "arms race" between external stressors impact, fitness costs and genetic adaptation by the bacteria, unravelling a non-linear relationship between the tested variables and the antimicrobial susceptibility of *Aeromonas* spp.

5.6. Conclusions

Current results show how *Aeromonas* spp. will respond to projected environmental shifts in water temperature and pH. Namely, that temperature increments will have a biphasic effect on *Aeromonas* spp. growth, while this bacterial genus will multiply better in acidic environments. Further, *Aeromonas* spp. biofilm production will be decreased due to temperature oscillations and microbial interactions in mixed cultures. Finally, antimicrobial resistance signatures of *Aeromonas* spp. will vary individually to changing temperature and pH parameters. Although general patterns were observed, it is evident that modulation of the intrinsic bacterial characteristics varies across isolates and that the final expression pattern will be influenced by environmental drivers and individual variability; however, the general patterns determined with this study deepen our knowledge on bacterial alterations expected in aquatic environments, strengthening our awareness and response to future bacterial outbreaks and how to deal with them.

Simplification of experimental settings, such as the approach applied in this study, has the limitation of disregarding the role of many other biotic and abiotic factors that can play a role in bacterial growth and virulence and resistance expression. Additionally, focusing on one bacterial genus to study such interactions is a major limitation of this study, since it fails to represent both the outcomes of a bacterial community that closely

resembles natural communities, as well as beneficial and detrimental effects of distinct bacterial strains/species on a particular bacterial strain in focus. Further development of microcosm experiments to accommodate more complex networks of drivers and bacterial communities is required.

Chapter 6. Discussion, Conclusion and Future perspectives

Sub-Chapter 6.1. Discussion

Health assessments of wild fish populations without economic importance, concerning threatened Iberian leuciscids in particular, has been a neglected area of fish conservation (Maceda-Veiga 2013). Regarding threatened Iberian leuciscids, established investigations often focused on pathogens prevalence alone, without establishing health parameters for the host species, describing pattern differences between populations/species or understanding the dynamics associated with the host-pathogen interaction (Bueno 1980; Teixeira et al. 2018; Rocha et al. 2019; Dias et al. 2020; Benovics et al. 2021). Furthermore, several studies relied on euthanasia of the hosts to perform such investigations, a technique considered unsustainable when working with imperilled species (Santos et al. 2019, 2021).

While investigating topics related to fish population health with important pathogenic agents, active surveillance schemes are also opportunistic platforms to unravel antimicrobial resistance and virulence prevalence in bacterial indicator species and to disclose natural reservoirs with potential impacts at the One Health level (Vittecoq et al. 2016).

Additionally, and since translocation into captivity and *ex situ* breeding programs are common and useful tools in the conservation of threatened Iberian leuciscids (Sousa-Santos et al. 2014b), it is essential to develop biosafety protocols that help to decrease potential pathogens prevalence, pathogenicity and dissemination across the program constituents (i.e., fishes in the program, handlers, other animals in the institution), at the same time that try to tackle antimicrobial resistance acquisition and dissemination among different landscapes.

Finally, to understand bacterial responses in terms of antimicrobial resistance and virulence profiles to projected climatic scenarios, and especially in regions where environmental alterations are expected to be particular evident and antimicrobial resistance accumulation is expected to be high (Reverter et al. 2020), such as the Iberian Peninsula, it is essential to perceive the interaction scenario in which threatened Iberian leuciscids and potential pathogenic agents will co-exist.

In this PhD thesis, a focus on the interaction between mesophilic *Aeromonas* spp. and *I. lusitanicum* (and *S. pyrenaicus* to a lesser extent) was made in order to generate important information that can be of assistance in conservation management planning of threatened Iberian leuciscids, as well as important surveillance markers for Health agencies of antimicrobial resistance and virulence determinants in freshwater streams from the Lisbon district.

6.1.1. Mesophilic *Aeromonas* in threatened Iberian leuciscids

For the first time, we conducted a bacterial survey regarding an important freshwater fish pathogenic genus – *Aeromonas* – in the critically endangered *I. lusitanicum*. We support past concerns from other authors in relationship to the dry season being a critical time period for threatened Iberian leuciscids (Maceda-Veiga 2013; Sousa-Santos et al. 2016), by demonstrating that fishes experience higher prevalence and extension of skin lesions during this season, while presenting a trend of poorer condition score (without statistical evidence). Origin of the populations, and hence the habitat to each they are exposed, also influences this pattern, reinforcing the idea that individual populations of threatened Iberian leuciscids need to be addressed as Operational Conservation Units (OTU's) (Robalo et al. 2007b; Sousa-Santos et al. 2016) regarding their health status as well, a consideration that should be mandatory in their management plans. Furthermore, our findings support evidence regarding juveniles' susceptibility to adverse conditions (Wang et al. 2008; Cornet et al. 2020) by concluding that body condition is lower and extension of skin lesions is higher in this group than in adults, raising concern regarding juveniles' survival, recruitment and future sustainability of the species.

Although causality between skin lesions and mesophilic *Aeromonas* spp. could not be proven in our study, we demonstrated that structure in the *Aeromonas* communities associated with *I. lusitanicum* individuals is dependent of the origin and of seasonality, and furthermore we show that the predominance of species considered more pathogenic (i.e., *A. hydrophila*) is stronger in the dry season, being replaced to an extent in the wet season. This type of phenomenon (i.e., species predominance) is common in bacterial infections, where pathogenic strains tend to be overexpressed and decrease bacterial diversity (Rahman et al. 2002; Hossain et al. 2014; Rasmussen-Ivey et al. 2016b). Additionally, *A. veronii* high prevalence was associated with the population with the highest extension of skin lesions and the lowest body condition (i.e., Samarra in the dry season). Our results support building evidence for the role of this species as a primary pathogen (Ran et al. 2018), rather than an opportunistic one, and suggest specific epidemiological scenarios in Portuguese freshwater habitats that do not correspond to the majority of reported cases (Cipriano 2001; Smith et al. 2019).

We also showed that sympatric threatened Iberian leuciscids, despite being influenced by similar environmental conditions, can display different levels of skin lesions' extension and that higher levels of skin lesion are accompanied by specific *Aeromonas* clusters that have higher pathogenic potential. Such results support the evidence that host-microbiome associations are influenced by specific factors, such as the host's genetic background (Smith et al. 2015; Steury et al. 2019), and that a further knowledge regarding inter-species

susceptibility to pathogenic agents is needed in order to identify populations at higher risk of developing infections and prioritize conservation managements to counteract the effects such infections could have in the sustainability of the population (Figure 32).

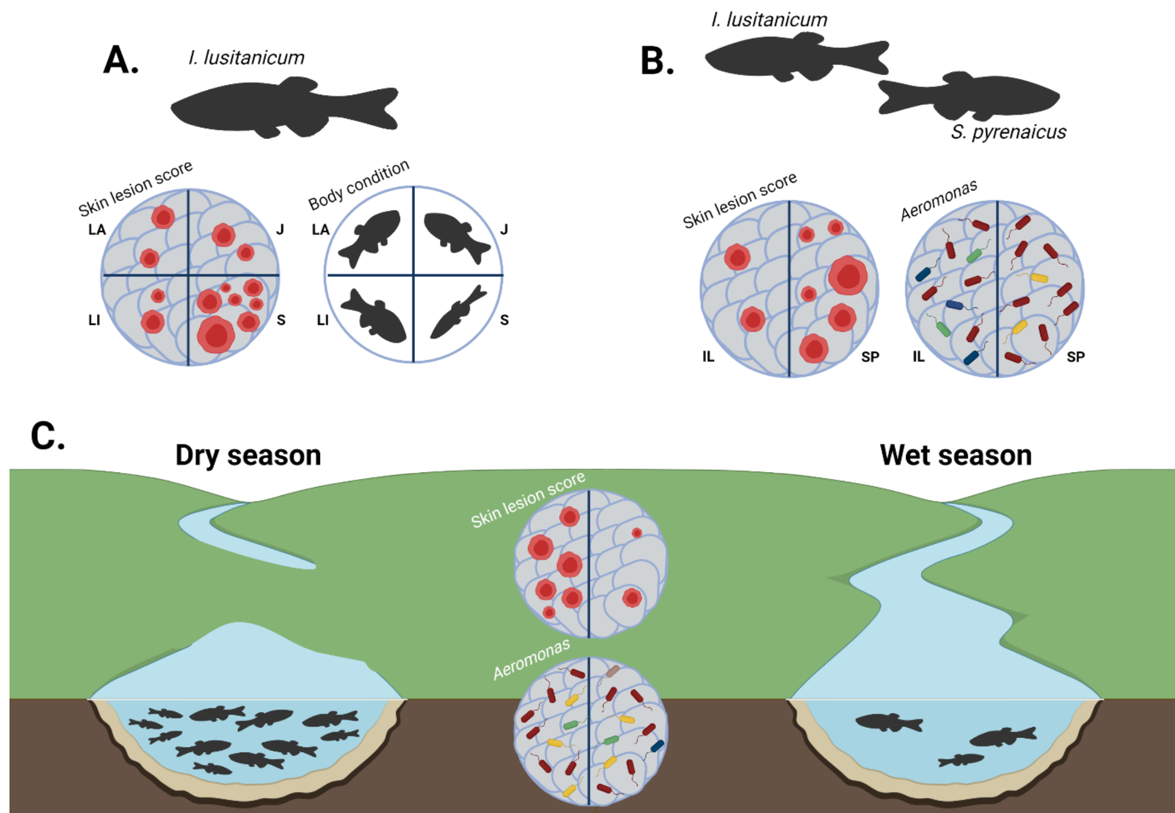

Figure 292. Schematic results of chapter 2 (A and C) and chapter 3 (B). LA – Laje, LI – Lizandro, J- Jamor, S – Samarra. Created with BioRender.com.

6.1.2. Wildlife reservoirs of antimicrobial resistance and virulence determinants in mesophilic *Aeromonas*

Despite the fact that surveillance of antimicrobial resistance and virulence determinants has become more common in aquatic ecosystems, and particularly in Portugal (Tacão et al. 2015; Varela et al. 2016; Gomes et al. 2021), following recognition of these habitats as reservoirs of important determinants at the One Health level (Fletcher 2015; Chen et al. 2018), we showed for the first time the role of small freshwater streams in the Lisbon district area as reservoirs of antimicrobial resistance and virulence determinants. Furthermore, we exposed how wild populations of *I. lusitanicum* are colonised by microbiota harbouring such determinants and provided evidence for their role as reservoirs in the wild. It is noteworthy that such streams are in close association with anthropogenic settings, often being

used for a variety of activities (e.g., agriculture, recreational), contributing to a close contact with these populations and creating interaction bridges that culminate in antimicrobial resistance and virulence acquisition by the human and domestic animal populations.

The prevalence of virulence factor's activity by the isolates collected in this study, and hence their pathogenic potential, was higher in the dry season. Not only this result constitutes a concern for the *I. lusitanicum* populations homeostasis, already experiencing severe adverse environmental conditions during this period, but is also alarming for human and domestic animals in close contact with these streams, as in this season their interaction with aquatic habitats may increase (i.e., irrigation necessities, fluvial beaches), consequently being more exposed to strains with higher pathogenic potential.

Our results suggest that the MAR index of the collected isolates and the prevalence of multidrug resistant strains is highly influenced by seasonality, exposing the wet season as a period for higher accumulation of antimicrobial resistance in mesophilic *Aeromonas* spp. from *I. lusitanicum*. We also observed lower multidrug resistant prevalence than in other studies conducted with *Aeromonas* spp. (Perretta et al. 2018; Borella et al. 2020), including in other regions of Portugal (Gomes et al. 2021). Our results suggest a lower selective pressure in the surveyed streams in comparison with other locations. However, it is noteworthy that we still found considerable prevalence of resistance phenotypes associated with carbapenem and quinolone resistance. We showed that such phenotypes are established in wild *I. lusitanicum* populations, urging further investigations on sources of antimicrobial resistance determinants and antimicrobial compound introduction in small riverine streams such as the studied ones, as well as in the establishment of prevention and educational measures to decrease posterior dissemination to the human and domestic animal populations (Figure 33).

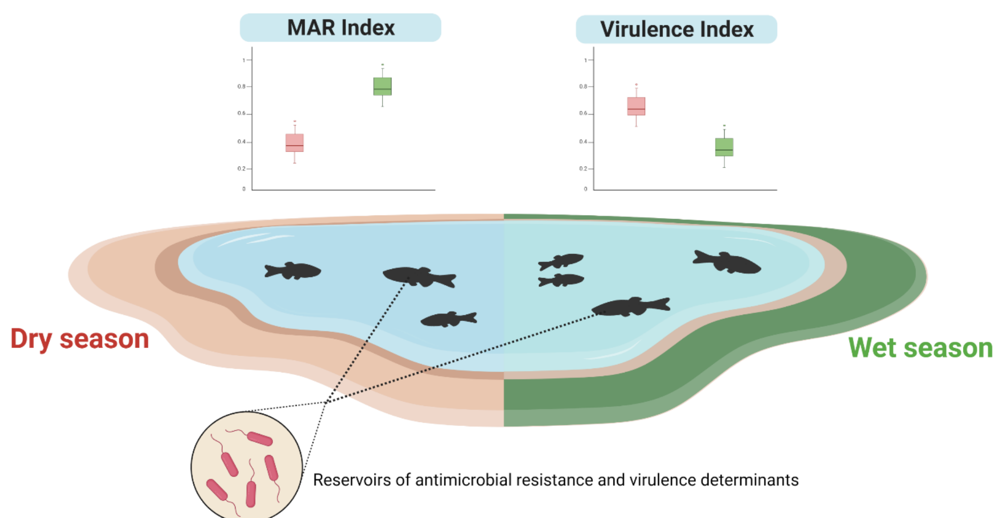

Figure 30. Schematic results of chapter 2. Created with BioRender.com.

6.1.3. Biosafety protocols for mesophilic *Aeromonas* in *ex situ* breeding programs for threatened Iberian leuciscids

Although some work regarding *ex situ* programs, biosafety measures and antimicrobial resistance acquisition has been developed in the past with terrestrial species (Stoddard et al. 2009; Power et al. 2013), we have implemented the first experimental investigation in *ex situ* program for aquatic species.

Despite the technical limitations existing prior to study, such as sample size, fish characteristics and infrastructures available, hampering the causality link between studied measures and the observations made, our results suggested that the use of simple protective measures has the potential to decrease the prevalence of mesophilic *Aeromonas* spp., retain an *Aeromonas* community more closely resembling the one present in the natural habitat and decrease the pathogenic potential of *Aeromonas* isolates to *I. lusitanicum* in the breeding program.

Our results also showed how husbandry techniques associated with the *ex situ* program can be a vehicle for mesophilic *Aeromonas* spp. acquisition by aquarists working in this field. The recognition of the *Aeromonas* genus as emergent pathogens is widely acknowledged (Fernández-Bravo and Figueras 2020), and professionals working with aquatic species are considered at risk of developing infections with these bacterial species, especially when presenting skin abrasions or cuts (Lehane and Rawlin 2000; Lowry and Smith 2007). A change of behaviours is urged in the husbandry of aquatic species in *ex situ* programs, not only regarding personal health safety for handlers, but also to prevent dissemination of pathogenic agents across different background animals.

The studied biosafety measures were not successful in preventing antimicrobial resistance acquisition in the *ex situ* program. In fact, similar to findings reported by previous studies (Stoddard et al. 2009; Power et al. 2013), we showed that prevalence of multidrug resistant isolates was high along the period of the experiment and that the MAR index values of the isolates in both conditions were higher in end of the experiment when compared to the beginning. We suggest a common route of antimicrobial resistance determinants (i.e., water introduction) in both tanks. Our findings suggest that the further relocation of *I. lusitanicum* individuals into their natural habitat can be met with the introduction of new antimicrobial resistance determinants, which raises concern on the role of these actions in promoting new antimicrobial resistance reservoirs in Portuguese freshwater habitats. Despite this fact and the need to continue pursuing methodologies that prevent antimicrobial resistance acquisition and dissemination by *ex situ* programs, their value as a last resource in the conservation of threatened Iberian leuciscids needs to be taken in consideration when evaluating pros and cons (Figure 34).

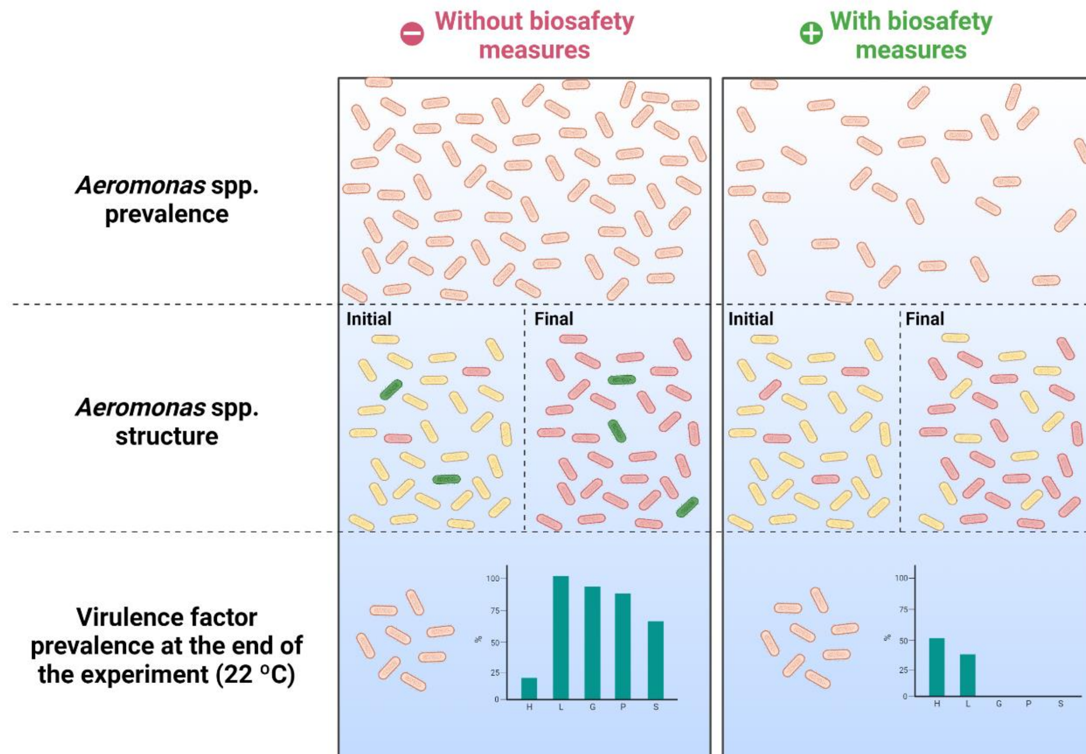

Figure 31. Schematic results of chapter 4. Created with BioRender.com.

6.1.4 Growth, biofilm production and antimicrobial resistance responses of mesophilic *Aeromonas* to climacteric alterations

We explored, for the first time, the effects that future environmental projections have in mesophilic *Aeromonas* spp. growth, biofilm production and antimicrobial resistance signatures, and showed that future climatic scenarios will influence these species proliferation ability, as well as their capability of producing biofilm and their antimicrobial resistance profiles. However, we also showed that, despite conclusions can be drawn for the tested species as a group, strain variability is evident.

Our results suggest that mesophilic *Aeromonas* spp. will benefit from small temperature increments, increasing their growth potential, while greater increments will reduce it. Furthermore, their growth will be higher in acidic aquatic environments. It is noteworthy that these changes will coincide with the warmer months of the year, a period already considered critical for *I. lusitanicum* populations (Maceda-Veiga 2013), and that a higher proliferation of mesophilic *Aeromonas* spp. will enhance the contact and colonization of these individuals. Although we did not find a correlation between higher mesophilic aeromonads and the extension of skin lesions in *I. lusitanicum* and in *S. pyrenaicus* in our previous work, another study explored this relationship and suggested the potential risk for infection development with higher loads (Samayanpaulraj et al. 2019).

Despite this, we also found evidence that biofilm production in mesophilic *Aeromonas* spp. will be affected by temperature oscillations, a phenomenon expected to increase with climatic alterations (Saarinen et al. 2019). These finding suggests mesophilic *Aeromonas* spp. will likely experience difficulties in expressing their virulence mechanisms under harsher environmental conditions, but a likely rapid bacterial evolution in response to continued environmental stimulus can also indicate that longer exposure periods that those tested in this work can result in a bacterial adaptation. This consideration is similar to the antimicrobial resistance phenotypic expression, which can be prompted or silenced with a variety of combinations of the tested treatments of environmental parameters as evidenced by our results. Nevertheless, these findings provide valuable knowledge on future responses of mesophilic *Aeromonas* spp. to climatic alterations, that need to be taken into consideration in long-term management of threatened Iberian leuciscids (Figure 35).

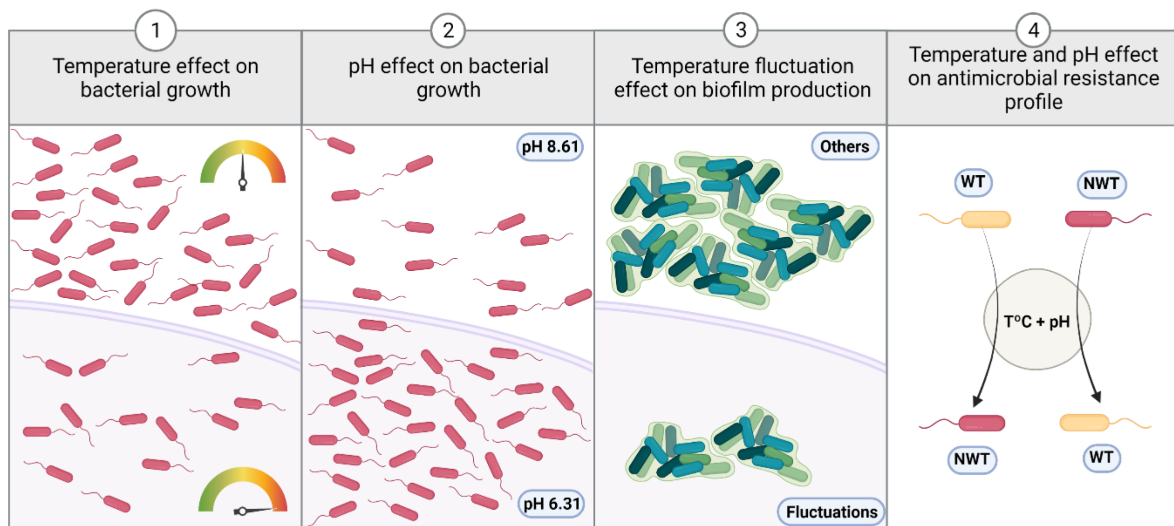

Figure 32. Schematic results of chapter 5. WT- wild-type; NWT – non-wild-type. Created with BioRender.com.

Sub-Chapter 6.2. Conclusions

The findings exposed in this thesis contribute to our knowledge on the interaction between mesophilic *Aeromonas* spp. and threatened Iberian leuciscids. Conservation Medicine and active surveillance approaches need to be addressed as fundamental parts of any successful conservation program for a species at risk of extinction. Health assessments are crucial management evaluation tools since changes in a population homeostasis can inform us on imbalances occurring between individuals and the habitat they occupy. Furthermore, they can be considered additional ecological indicators of environmental quality, complying with international legislation requiring such interventions (e.g., Water Framework Directive in Europe). In addition to health evaluations of wild populations, guaranteeing the safety associated with the translocation of this species into captivity is essential and constant efforts need to be made in order to decrease pathogen presence and virulence in *ex situ* programs, as well as to decrease possible deleterious effects associated with these actions. Finally, unravelling bacterial responses to future environmental scenarios further increases our prediction power towards host-pathogen interactions, hence making possible the creation of more robust conservation management plans. Decision making based on informed scientific data increases the probability of success of conservation programs for threatened Iberian leuciscids (Figure 36).

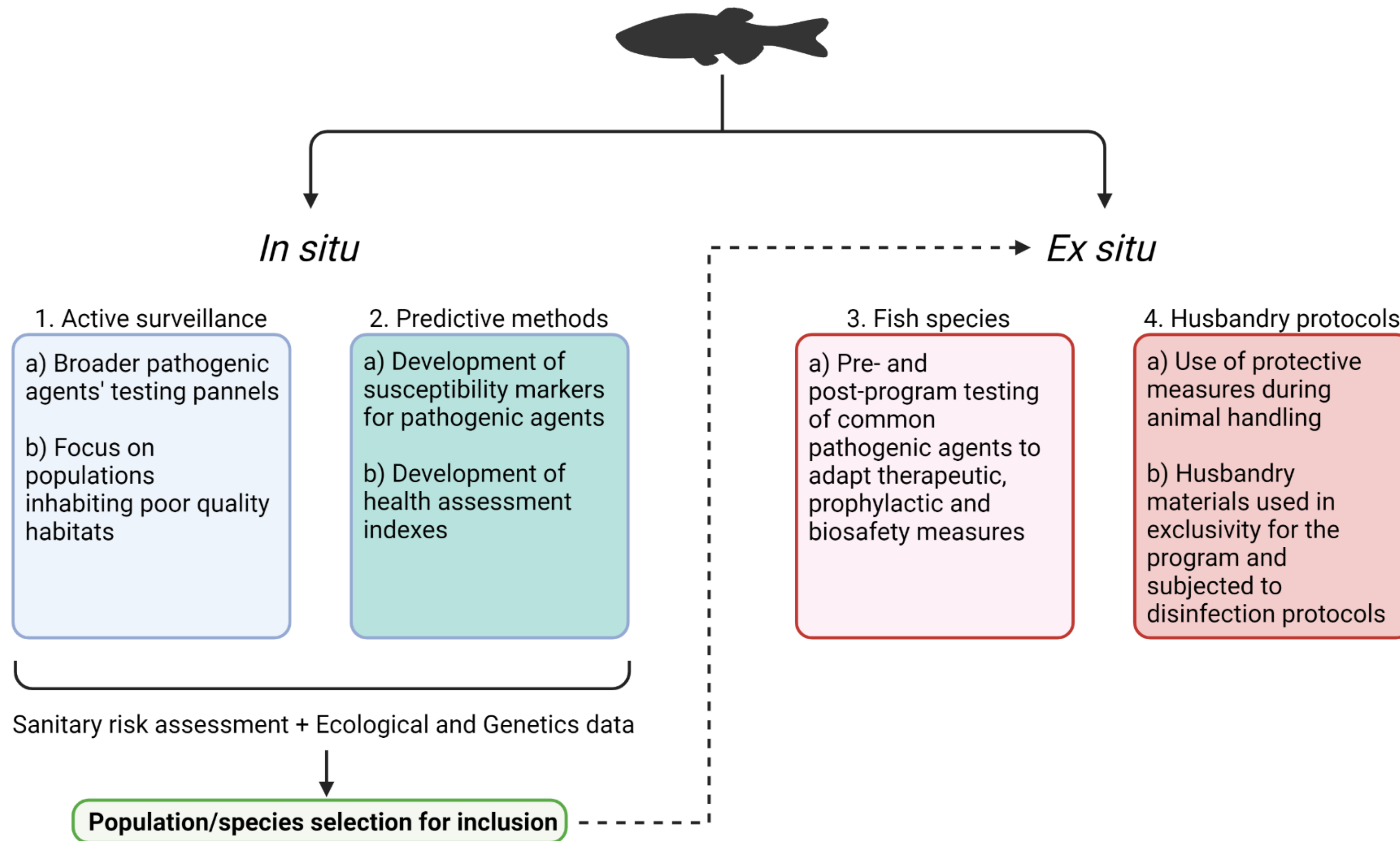

Figure 33. Health conservation recommendations to be included in the Conservation programs of threatened Iberian leuciscids. Created with BioRender.com.

Sub-Chapter 6.3. Future perspectives

Although the advances obtained with this thesis, several knowledge gaps still exist that should be taken into consideration in further research.

Despite our methodological approach relied on simplification by choosing a model pathogen and host, the lack of knowledge regarding the impact and prevalence of several infectious agents is still evident in threatened Iberian leuciscids. Namely, bacterial and viral pathogens are underrepresented in performed investigations when compared to parasitic infections. A broader testing panel designed for freshwater fishes should be implemented in order to understand the contribution of each pathogenic agent in specific locations and time periods to the homeostasis of these species, such as the investigation of *Aeromonas salmonicida*, *Flavobacterium* spp., *Renibacterium salmoninarum*, *Edwardsiella* spp., *Mycobacterium* spp., *Pseudomonas* spp., *Vibrio* spp., *Shewanella putrefaciens*, *Streptococcus iniae*, *Yersinia ruckeri,* Viral Hemorrhagic Septicemia virus, Spring Viremia of Carp virus and ranavirus, among others. Similarly, and since our results point towards differences in skin lesion extension between distinct fish species, other threatened Iberian leuciscid species should be included in the surveillance schemes, as well as other species present in the natural habitat that can act as disease reservoirs. Conservation programs would also benefit from the development of non-lethal marker methodologies that would identify susceptibility differences to infectious agents among different populations/species, in order to prioritize conservation measures. Knowledge acquired in aquaculture research regarding genetic markers, such as polymorphisms in the major histocompatibility complex, could be a starting point. Additionally, although difficult to execute due to unpredictability, investigation of mass mortalities should be conducted to determine definitive cases of infection and mortality causes when possible.

Regarding antimicrobial resistance and virulence prevalence in natural habitats, and since their eradication of natural habitats is virtually impossible, efforts need to be directed into disclosing entrance points for specific streams, which can be facilitated by the use of microbial source tracking techniques. Also, and similar to *ex situ* breeding programs, remediation treatments that eliminate antimicrobial resistance and virulence determinants in drinking water are required. However, scientific investigations need to be accompanied by implementation and supervision policies that guarantee that entrance routes are discontinued and important stakeholders (i.e., industry, sewage plants and agricultural practices) comply with available legislation preventing illegal discharges into freshwater streams.

Ex situ breeding programs for threatened Iberian leuciscids would benefit from a wider knowledge of the epidemiological scenario in natural habitats, in order to adapt husbandry techniques to the health status of the target population but also in preventing pathogen spill-over to distinct populations/species possible naïve to infectious agents common in the original population. Despite the suggested potential of the biosafety measures studied here, it is important to evaluate their efficacy regarding other important and common pathogenic agents in freshwater fishes. Furthermore, similar to studies conducted in terrestrial species, it is fundamental to investigate changes in the microbiome of individuals kept in captivity, their possible impact in the individual homeostasis, in the breeding success and in the future relocation in natural habitats.

Finally, when recreating natural habitats and evaluating bacterial responses to environmental drivers, it is essential to adapt microcosm experiments to the complexity experienced in the aquatic ecosystems. Namely, future investigations should aim at evaluating bacterial communities representing the diversity found in natural environments, as well as to test the role of other drivers (e.g., nutrient availability, mobile genetic elements abundance) in promoting antimicrobial resistance and virulence expression.

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Annexes

Annex I

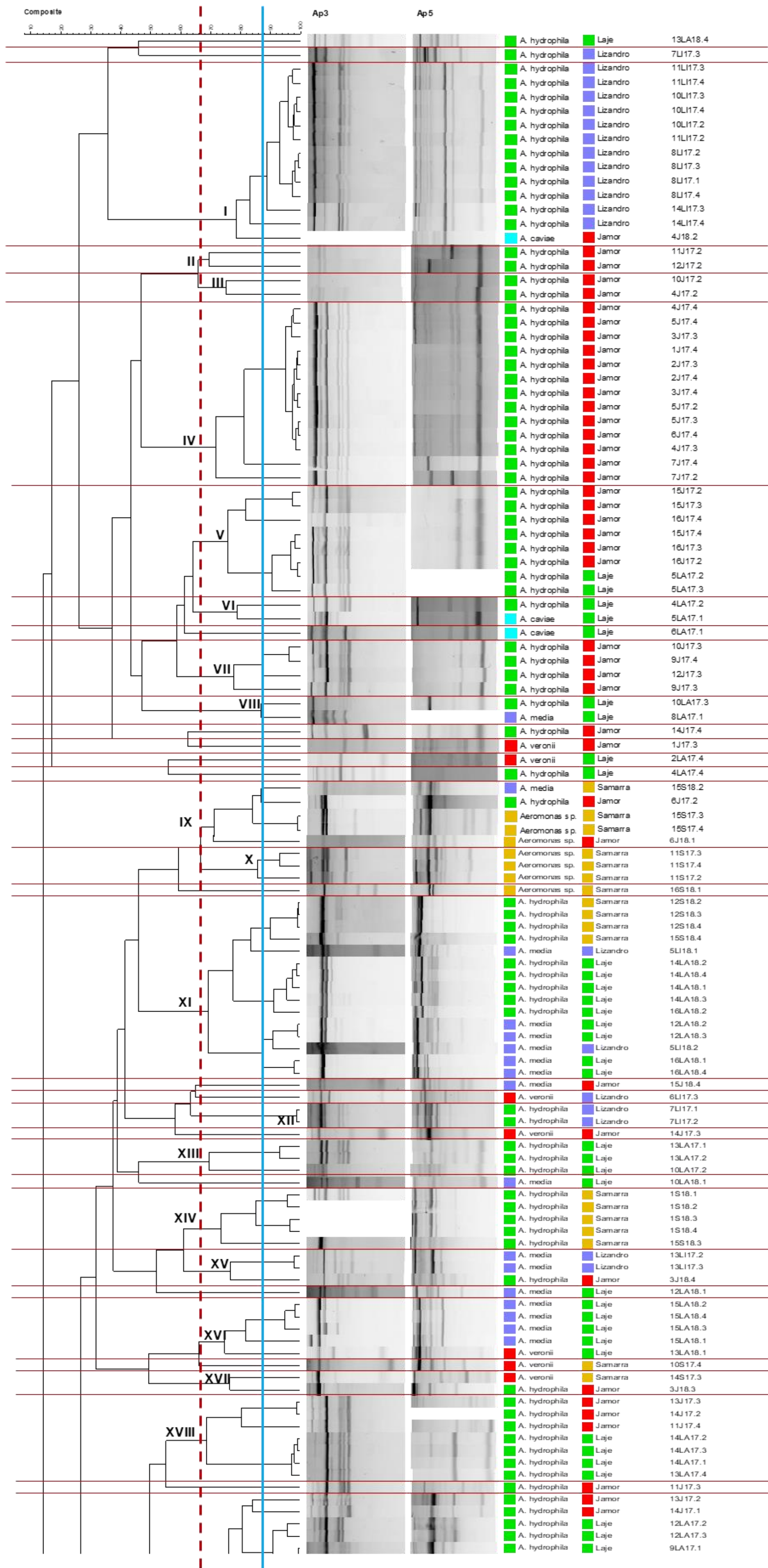
Supplementary Table S1. Parasitic and fungal agents' prevalence studies in threatened Iberian leuciscids.

Agent	Host	Reference
	Mussels	
Anodonta anatine	*Squalius alburnoides* *Pseudochondrostoma duriense*	Teixeira et al. 2018; Dias et al. 2020
Unio sp.	*Squalius alburnoides* *Achondrostoma arcasii* *Pseudochondrostoma duriense*	Illán 2012
Unio delphinus	*Pseudochondrostoma duriense* *Squalius alburnoides*	Teixeira et al. 2018
Unio tumidiformis	*Squalius pyrenaicus*	Reis et al. 2014
Potomida littoralis	*Pseudochondrostoma duriense* *Squalius alburnoides*	Teixeira et al. 2018
	Copepods	
Ergasilus sieboldi	*Pseudochondrostoma duriense*	Illán 2012
Lernaea cyprinacea	*Pseudochondrostoma duriense* *Pseudochondrostoma willkommii*	Perez-Bote 2000; Illán 2012; Sánchez-Hernández 2017
	Trematodes	
Allocreadium isosporum	*Squalius alburnoides*	Simon Vicente et al. 1973
Asymphylodora tincae	*Achondrostoma arcasii* *Squalius pyrenaicus*	Bueno 1980
Dactylogyrus sp.	*Achondrostoma arcasii* *Achondrostoma occidentale* *Iberochondrostoma almacai* *Squalius alburnoides* *Squalius aradensis* *Squalius pyrenaicus* *Squalius torgalensis* *Parachondrostoma turiense* *Pseudochondrostoma duriense*	Illán 2012; Benovics et al. 2020
Dactylogyrus polylepidis	*Achondrostoma arcasii* *Pseudochondrostoma duriense*	Benovics et al. 2020
Dactylogyrus elegantis	*Achondrostoma arcasii*	Simon Vicente et al. 1973
Dactylogyrus ergensi	*Achondrostoma arcasii*	Álvarez-Pellitero et al. 1981
Gyrodactylus sp.	*Achondrostoma arcasii* *Pseudochondrostoma duriense*	Simon Vicente, 1975; Illán, 2012

Gyrodactylus medius	*Squalius pyrenaicus*	Simon Vicente and Ramajo Martin 1971
Hysteromorpha triloba	*Pseudochondrostoma duriense*	Illán et al. 2013
	Iberochondrostoma lusitanicum	
	Squalius pyrenaicus	
Paradiplozoon sp.	*Squalius valentinus*	Čermáková et al. 2018
	Parachondrostoma arrigonis	
	Parachondrostoma turiense	
Paradiplozoon homoion	*Achondrostoma arcasii*	Čermáková et al. 2018; Benovics et al. 2021
	Iberochondrostoma lusitanicum	
	Parachondrostoma arrigonis	
Paradiplozoon ibericus	*Parachondrostoma turiensis*	Benovics et al. 2021
	Squalius pyrenaicus	
	Squalius valentinus	
	Cestodes	
Archigetes sp.	*Squalius pyrenaicus*	Bueno 1980
Caryophyllaeus laticeps	*Squalius pyrenaicus*	Bueno 1980
Ligula intestinalis	*Squalius pyrenaicus*	Martinez Gomes 1970
Khawia sp.	*Squalius pyrenaicus*	Bueno 1980
	Nematodes	
Cystidicoloides tenuissima	*Achondrostoma arcasii*	Bueno 1980
Rhabdochona denudata	*Achondrostoma arcasii* *Squalius pyrenaicus*	Bueno 1980
	Myxosporeans	
Myxobolus sp.	*Achondrostoma arcasii* *Pseudochondrostoma duriense*	Illán 2012
Myxobolus arcasii	*Achondrostoma arcasii*	Rocha et al. 2019
Myxobolus duriensis	*Pseudochondrostoma duriense*	Rocha et al. 2019
Myxobolus leuciscini	*Achondrostoma arcasii*	Gonzalez-Lanza and Alvarez-Pellitero 1985
Myxobolus muelleri	*Achondrostoma arcasii*	Gonzalez-Lanza and Alvarez-Pellitero 1985
Myxobolus pseudodispar	*Achondrostoma arcasii* *Pseudochondrostoma duriense*	Gonzalez-Lanza and Alvarez-Pellitero 1985; Rocha et al. 2019
	Ciliates	
Ichthyophthirius multifiliis	*Achondrostoma arcasii* *Pseudochondrostoma duriense*	Illán 2012
	Fishes	
Petromyzon marinus	*Pseudochondrostoma duriense*	Silva et al. 2013
	Oomycetes	
Saprolegnia parasitica	*Pseudochondrostoma duriense*	Aller-Gancedo et al. 2016

Annex II

Supplementary Figure S1. Dendrogram of the entire bacterial collection based on the composite analysis of RAPD fingerprints with primers Ap3 and Ap5 (Pearson correlation coefficient and UPGMA clustering). Blue line represents reproducibility level (87.6%). Isolates displaying higher similarity levels were considered identical. Red dash line represents cluster level (66.98%). Red lines easy the visualization of the defined clusters. Cophenetic correlation coefficient was 0.87. First column represents *Aeromonas* species, second column the location from where the strain was isolated and third column the isolate's code.



Composite
Ap3
Ap5
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
XIII
XIV
XV
XVI
XVII
XVIII
A. hydrophila Laje 13LA18.4
A. hydrophila Lizandro 7LI17.3
A. hydrophila Lizandro 11LI17.3
A. hydrophila Lizandro 11LI17.4
A. hydrophila Lizandro 10LI17.3
A. hydrophila Lizandro 10LI17.4
A. hydrophila Lizandro 10LI17.2
A. hydrophila Lizandro 11LI17.2
A. hydrophila Lizandro 8LI17.2
A. hydrophila Lizandro 8LI17.3
A. hydrophila Lizandro 8LI17.1
A. hydrophila Lizandro 8LI17.4
A. hydrophila Lizandro 14LI17.3
A. hydrophila Lizandro 14LI17.4
A. caviae Jamor 4J18.2
A. hydrophila Jamor 11J17.2
A. hydrophila Jamor 12J17.2
A. hydrophila Jamor 10J17.2
A. hydrophila Jamor 4J17.2
A. hydrophila Jamor 4J17.4
A. hydrophila Jamor 5J17.4
A. hydrophila Jamor 3J17.3
A. hydrophila Jamor 1J17.4
A. hydrophila Jamor 2J17.3
A. hydrophila Jamor 2J17.4
A. hydrophila Jamor 3J17.4
A. hydrophila Jamor 5J17.2
A. hydrophila Jamor 5J17.3
A. hydrophila Jamor 6J17.4
A. hydrophila Jamor 4J17.3
A. hydrophila Jamor 7J17.4
A. hydrophila Jamor 7J17.2
A. hydrophila Jamor 15J17.2
A. hydrophila Jamor 15J17.3
A. hydrophila Jamor 16J17.4
A. hydrophila Jamor 15J17.4
A. hydrophila Jamor 16J17.3
A. hydrophila Jamor 16J17.2
A. hydrophila Laje 5LA17.2
A. hydrophila Laje 5LA17.3
A. hydrophila Laje 4LA17.2
A. caviae Laje 5LA17.1
A. caviae Laje 6LA17.1
A. hydrophila Jamor 10J17.3
A. hydrophila Jamor 9J17.4
A. hydrophila Jamor 12J17.3
A. hydrophila Jamor 9J17.3
A. hydrophila Laje 10LA17.3
A. media Laje 8LA17.1
A. hydrophila Jamor 14J17.4
A. veronii Jamor 1J17.3
A. veronii Laje 2LA17.4
A. hydrophila Laje 4LA17.4
A. media Samarra 15S18.2
A. hydrophila Jamor 6J17.2
Aeromonas sp. Samarra 15S17.3
Aeromonas sp. Samarra 15S17.4
Aeromonas sp. Jamor 6J18.1
Aeromonas sp. Samarra 11S17.3
Aeromonas sp. Samarra 11S17.4
Aeromonas sp. Samarra 11S17.2
Aeromonas sp. Samarra 16S18.1
A. hydrophila Samarra 12S18.2
A. hydrophila Samarra 12S18.3
A. hydrophila Samarra 12S18.4
A. hydrophila Samarra 15S18.4
A. media Lizandro 5LI18.1
A. hydrophila Laje 14LA18.2
A. hydrophila Laje 14LA18.4
A. hydrophila Laje 14LA18.1
A. hydrophila Laje 14LA18.3
A. hydrophila Laje 16LA18.2
A. media Laje 12LA18.2
A. media Laje 12LA18.3
A. media Lizandro 5LI18.2
A. media Laje 16LA18.1
A. media Laje 16LA18.4
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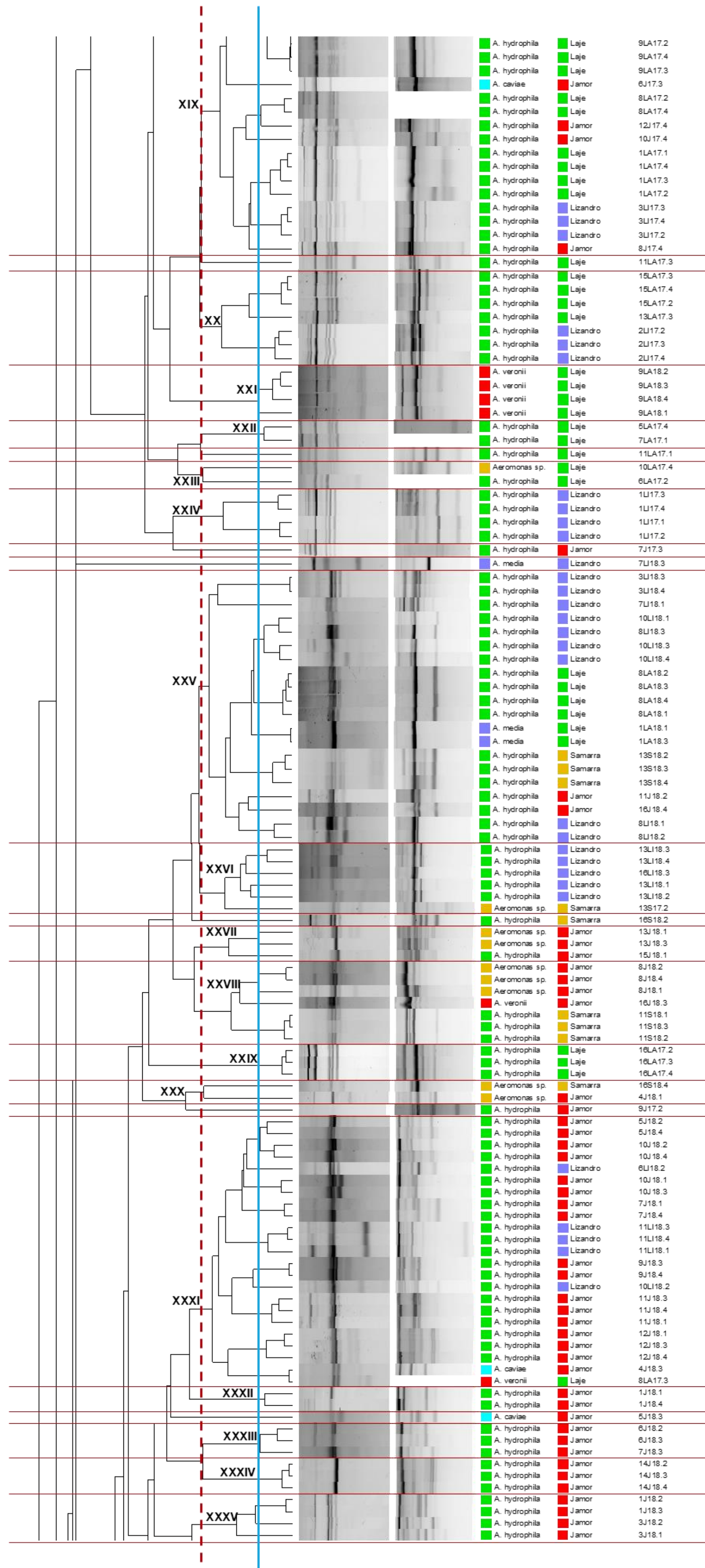
A. hydrophila Lizandro 7LI17.1
A. hydrophila Lizandro 7LI17.2
A. veronii Jamor 14J17.3
A. hydrophila Laje 13LA17.1
A. hydrophila Laje 13LA17.2
A. hydrophila Laje 10LA17.2
A. media Laje 10LA18.1
A. hydrophila Samarra 1S18.1
A. hydrophila Samarra 1S18.2
A. hydrophila Samarra 1S18.3
A. hydrophila Samarra 1S18.4
A. hydrophila Samarra 15S18.3
A. media Lizandro 13LI17.2
A. media Lizandro 13LI17.3
A. hydrophila Jamor 3J18.4
A. media Laje 12LA18.1
A. media Laje 15LA18.2
A. media Laje 15LA18.4
A. media Laje 15LA18.3
A. media Laje 15LA18.1
A. veronii Laje 13LA18.1
A. veronii Samarra 10S17.4
A. veronii Samarra 14S17.3
A. hydrophila Jamor 3J18.3
A. hydrophila Jamor 13J17.3
A. hydrophila Jamor 14J17.2
A. hydrophila Jamor 11J17.4
A. hydrophila Laje 14LA17.2
A. hydrophila Laje 14LA17.3
A. hydrophila Laje 14LA17.1
A. hydrophila Laje 13LA17.4
A. hydrophila Jamor 11J17.3
A. hydrophila Jamor 13J17.2
A. hydrophila Jamor 14J17.1
A. hydrophila Laje 12LA17.2
A. hydrophila Laje 12LA17.3
A. hydrophila Laje 9LA17.1



XIX
XX
XXI
XXII
XXIII
XXIV
XXV
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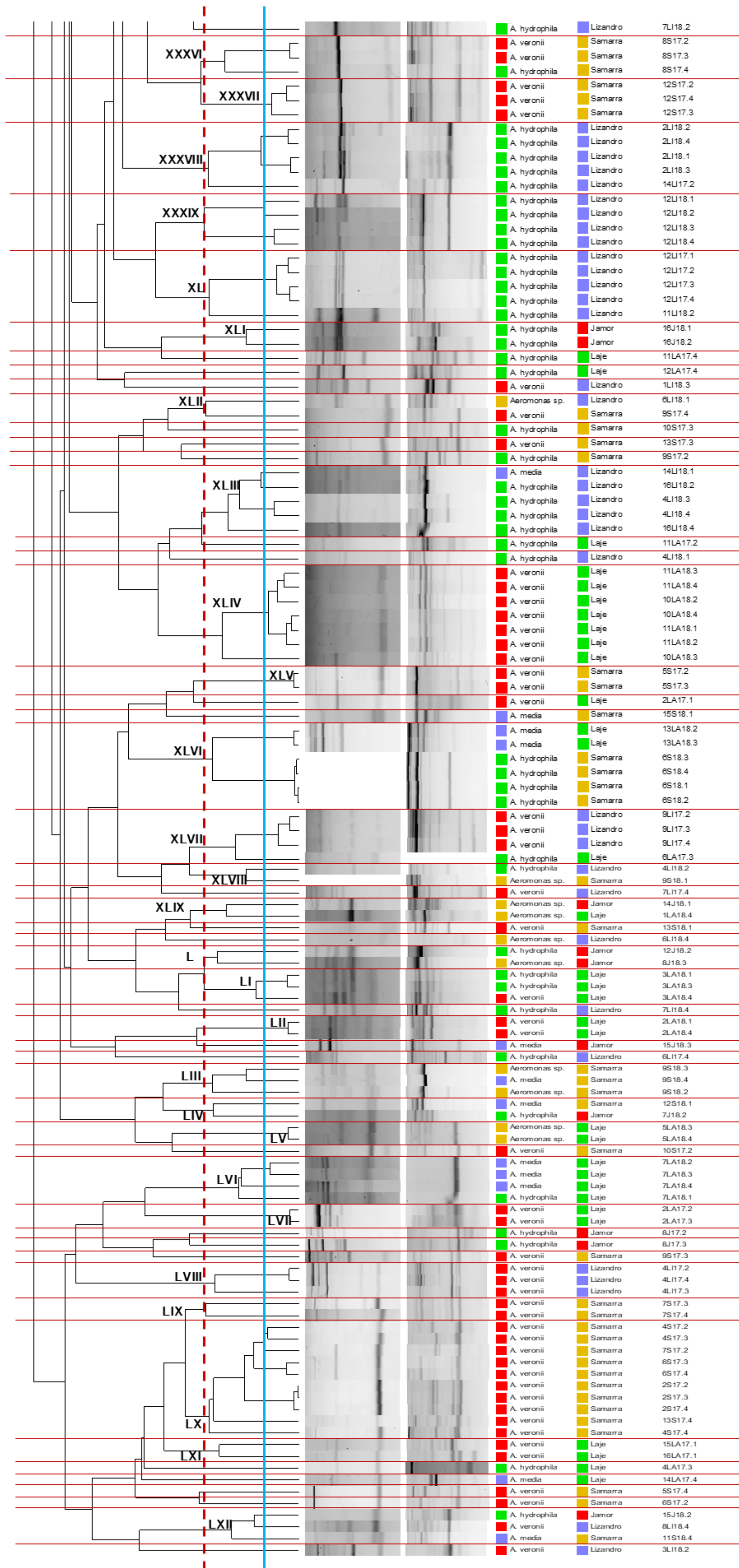
XXVIII
XXIX
XXX
XXXI
XXXII
XXXIII
XXXIV
XXXV



XXXVI
XXXVII
XXXVIII
XXXIX
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XLI
XLII
XLIII
XLIV
XLV
XLVI
XLVII
XLVIII
XLIX
L
LI
LII
LIII
LIV
LV
LVI
LVII
LVIII
LIX
LX
LXI
LXII
A. hydrophila Lizandro 7LI18.2
A. veronii Samarra 8S17.2
A. veronii Samarra 8S17.3
A. hydrophila Samarra 8S17.4
A. veronii Samarra 12S17.2
A. veronii Samarra 12S17.4
A. veronii Samarra 12S17.3
A. hydrophila Lizandro 2LI18.2
A. hydrophila Lizandro 2LI18.4
A. hydrophila Lizandro 2LI18.1
A. hydrophila Lizandro 2LI18.3
A. hydrophila Lizandro 14LI17.2
A. hydrophila Lizandro 12LI18.1
A. hydrophila Lizandro 12LI18.2
A. hydrophila Lizandro 12LI18.3
A. hydrophila Lizandro 12LI18.4
A. hydrophila Lizandro 12LI17.1
A. hydrophila Lizandro 12LI17.2
A. hydrophila Lizandro 12LI17.3
A. hydrophila Lizandro 12LI17.4
A. hydrophila Lizandro 11LI18.2
A. hydrophila Jamor 16J18.1
A. hydrophila Jamor 16J18.2
A. hydrophila Laje 11LA17.4
A. hydrophila Laje 12LA17.4
A. veronii Lizandro 1LI18.3
Aeromonas sp. Lizandro 6LI18.1
A. veronii Samarra 9S17.4
A. hydrophila Samarra 10S17.3
A. veronii Samarra 13S17.3
A. hydrophila Samarra 9S17.2
A. media Lizandro 14LI18.1
A. hydrophila Lizandro 16LI18.2
A. hydrophila Lizandro 4LI18.3
A. hydrophila Lizandro 4LI18.4
A. hydrophila Lizandro 16LI18.4
A. hydrophila Laje 11LA17.2
A. hydrophila Lizandro 4LI18.1
A. veronii Laje 11LA18.3
A. veronii Laje 11LA18.4
A. veronii Laje 10LA18.2
A. veronii Laje 10LA18.4
A. veronii Laje 11LA18.1
A. veronii Laje 11LA18.2
A. veronii Laje 10LA18.3
A. veronii Samarra 5S17.2
A. veronii Samarra 5S17.3
A. veronii Laje 2LA17.1
A. media Samarra 15S18.1
A. media Laje 13LA18.2
A. media Laje 13LA18.3
A. hydrophila Samarra 6S18.3
A. hydrophila Samarra 6S18.4
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A. veronii Lizandro 9LI17.3
A. veronii Lizandro 9LI17.4
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Aeromonas sp. Samarra 9S18.1
A. veronii Lizandro 7LI17.4
Aeromonas sp. Jamor 14J18.1
Aeromonas sp. Laje 1LA18.4
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Aeromonas sp. Lizandro 6LI18.4
A. hydrophila Jamor 12J18.2
Aeromonas sp. Jamor 8J18.3
A. hydrophila Laje 3LA18.1
A. hydrophila Laje 3LA18.3
A. veronii Laje 3LA18.4
A. hydrophila Lizandro 7LI18.4
A. veronii Laje 2LA18.1
A. veronii Laje 2LA18.4
A. media Jamor 15J18.3
A. hydrophila Lizandro 6LI17.4
Aeromonas sp. Samarra 9S18.3
A. media Samarra 9S18.4
Aeromonas sp. Samarra 9S18.2
A. media Samarra 12S18.1
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Aeromonas sp. Laje 5LA18.4
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A. media Laje 7LA18.3
A. media Laje 7LA18.4
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A. veronii Laje 2LA17.2
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A. hydrophila Jamor 8J17.3
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A. veronii Lizandro 4LI17.4
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A. veronii Samarra 7S17.4
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A. veronii Samarra 4S17.3
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A. veronii Samarra 6S17.4
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A. veronii Samarra 6S17.2
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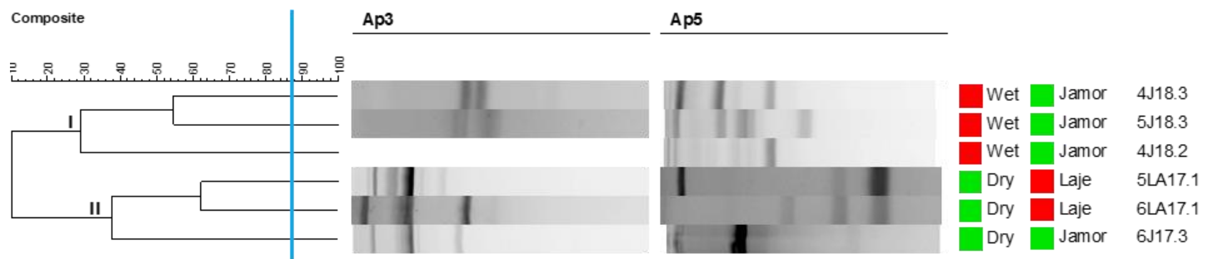
A. media Samarra 11S18.4
A. veronii Lizandro 3LI18.2

Annex III

Supplementary Figure S2. Dendrogram of *A. caviae* isolates based on the composite analysis of RAPD fingerprints with primers Ap3 and Ap5 (Pearson correlation coefficient and UPGMA clustering). Blue line represents reproducibility level (87.6%). Isolates displaying higher similarity levels were considered identical. Cophenetic correlation coefficient was 0.89. First column represents sampling season, second column the location from where the strain was isolated and third column the isolate's code.



Annex IV

Supplementary Figure S3. Dendrogram of *A. hydrophila* isolates based on the composite analysis of RAPD finger-prints with primers Ap3 and Ap5 (Pearson correlation coefficient and UPGMA clustering). Blue line represents reproducibility level (87.6%). Isolates displaying higher similarity levels were considered identical. Cophenetic correlation coefficient was 0.77. First column represents sampling season, second column the location from where the strain was isolated and third column the isolate's code.

I

II

III

IV

V

VI

VII

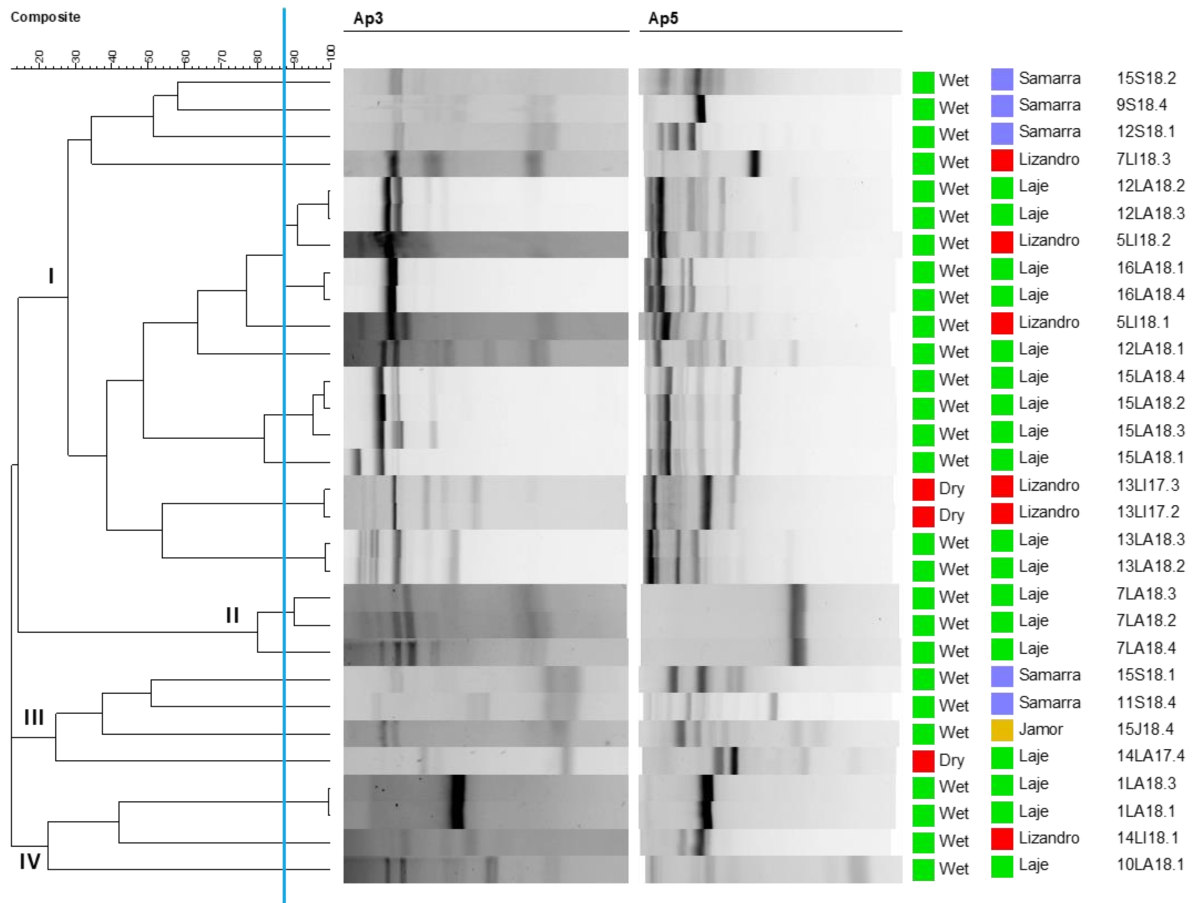
VIII

IX

X

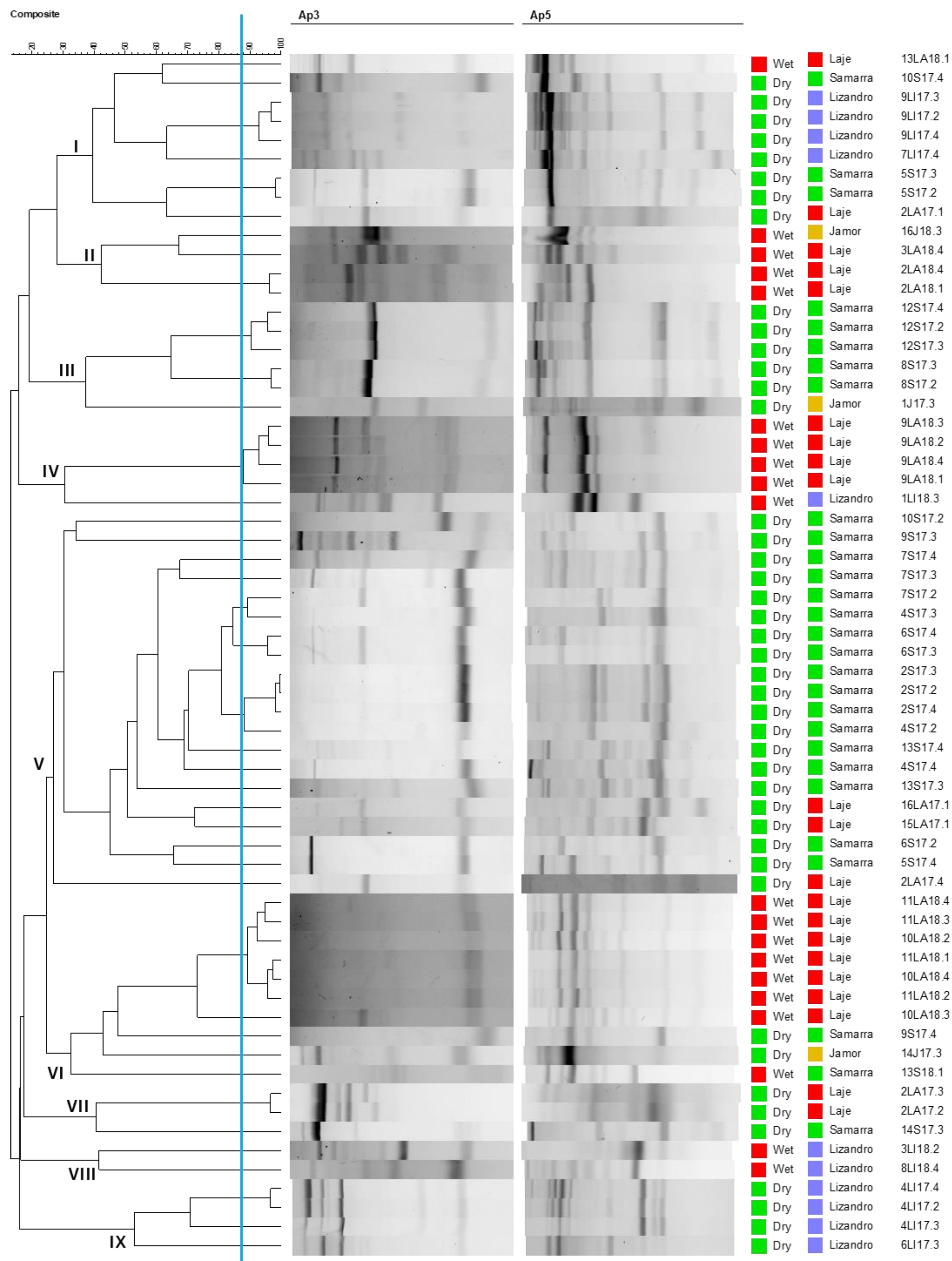
Annex V

Supplementary Figure S4. Dendrogram of *A. media* isolates based on the composite analysis of RAPD fingerprints with primers Ap3 and Ap5 (Pearson correlation coefficient and UPGMA clustering). Blue line represents reproducibility level (87.6%). Isolates displaying higher similarity levels were considered identical. Cophenetic correlation coefficient was 0.86. First column represents sampling season, second column the location from where the strain was isolated and third column the isolate's code.



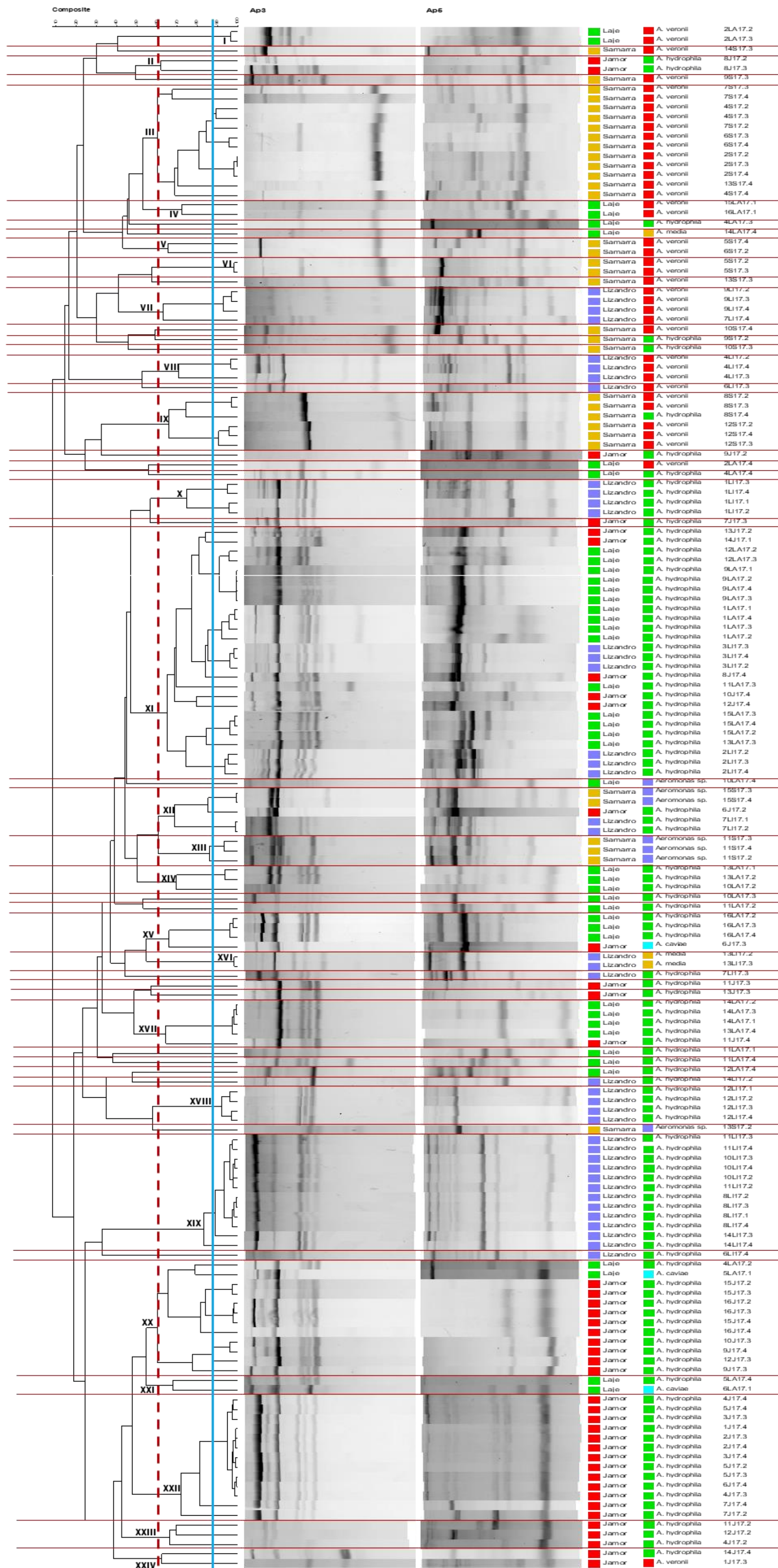
Annex VI

Supplementary Figure S5. Dendrogram of *A. veronii* isolates based on the composite analysis of RAPD finger-prints with primers Ap3 and Ap5 (Pearson correlation coefficient and UPGMA clustering). Blue line represents reproducibility level (87.6%). Isolates displaying higher similarity levels were considered identical. Cophenetic correlation coefficient was 0.82. First column represents sampling season, second column the location from where the strain was isolated and third column the isolate's code.



Annex VII

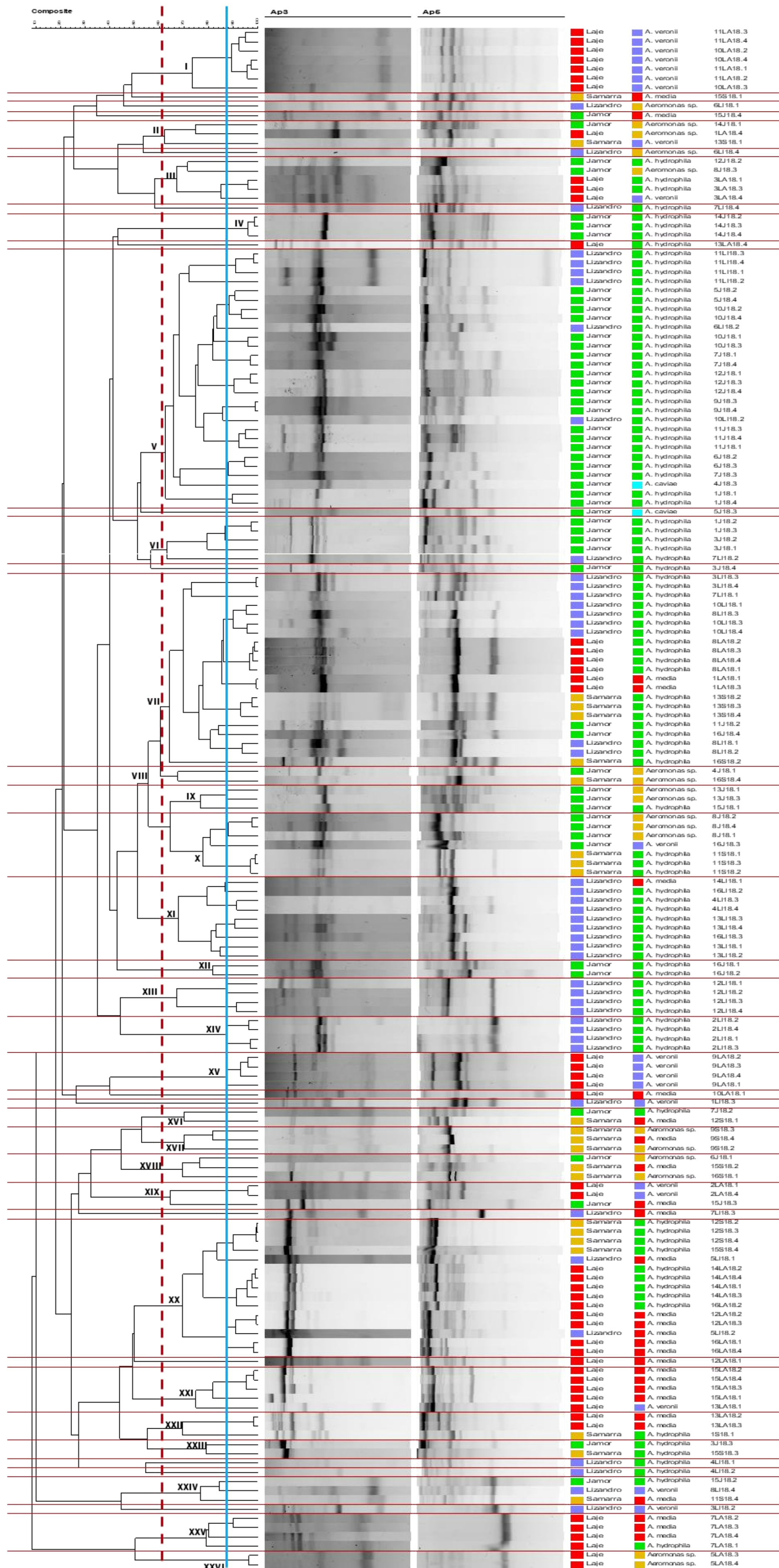
Supplementary Figure S6. Dendrogram of the isolates collected in animals sampled in the dry season based on the composite analysis of RAPD finger-prints with primers Ap3 and Ap5 (Pearson correlation coefficient and UPGMA clustering). Blue line represents reproducibility level (87.6%). Isolates displaying higher similarity levels were considered identical. Red dash line represents cluster level (60.46%). Red lines easy the visualization of the defined clusters. Cophenetic correlation coefficient was 0.83. First column represents sampling location, second column the *Aeromonas* species and third column the isolate's code.



Composite
Ap3
Ap6
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
XIII
XIV
XV
XVI
XVII
XVIII
XIX
XX
XXI
XXII
XXIII
XXIV

Annex VIII

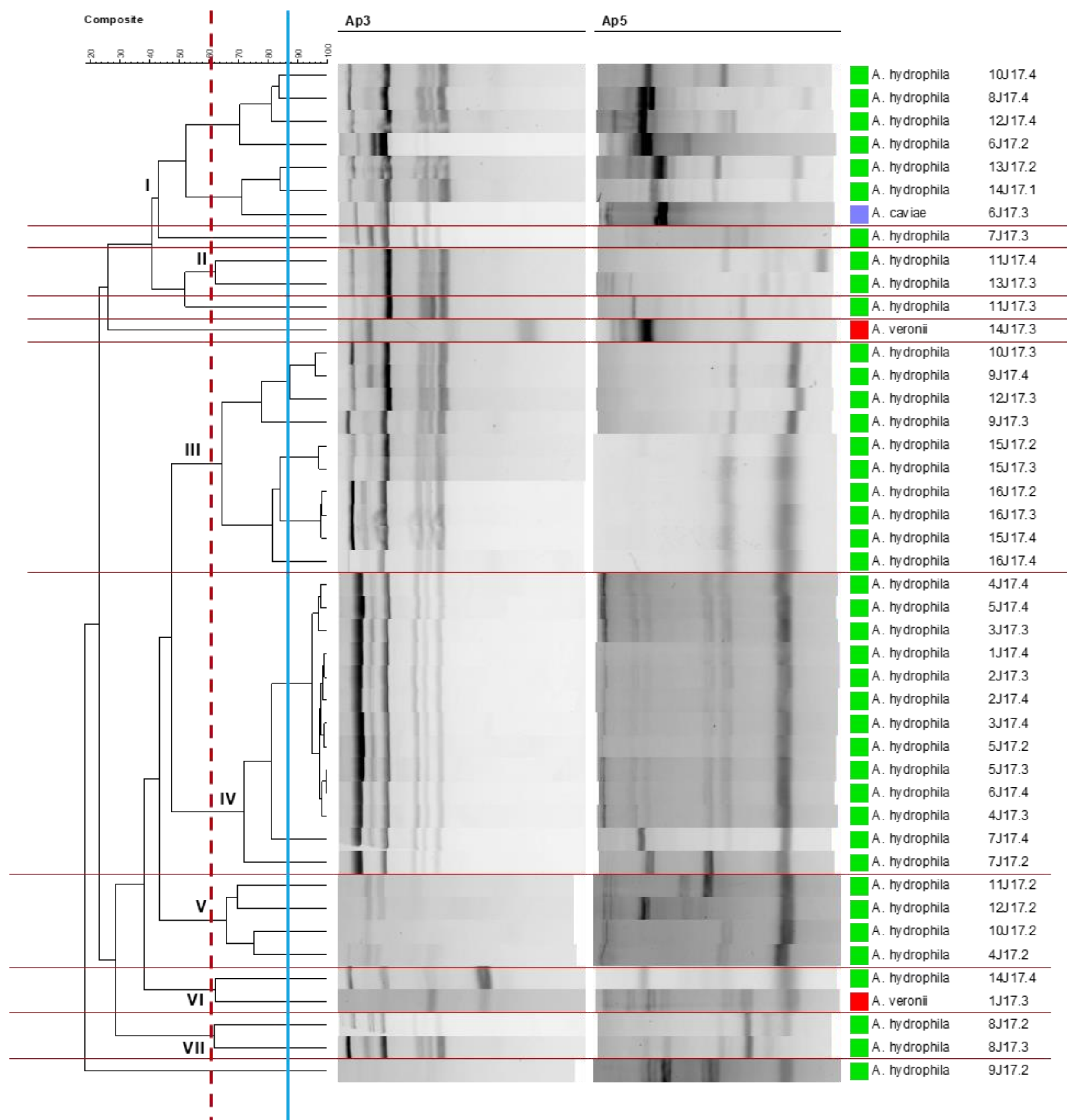
Supplementary Figure S7. Dendrogram of the isolates collected in animals sampled in the wet season based on the composite analysis of RAPD finger-prints with primers Ap3 and Ap5 (Pearson correlation coefficient and UPGMA clustering). Blue line represents reproducibility level (87.6%). Isolates displaying higher similarity levels were considered identical. Red dash line represents cluster level (60.46%). Red lines easy the visualization of the defined clusters. Cophenetic correlation coefficient was 0.79. First column represents sampling location, second column the *Aeromonas* species and third column the isolate’s code.



Composite
Ap3
Ap6
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
XIII
XIV
XV
XVI
XVII
XVIII
XIX
XX
XXI
XXII
XXIII
XXIV
XXV
XXVI

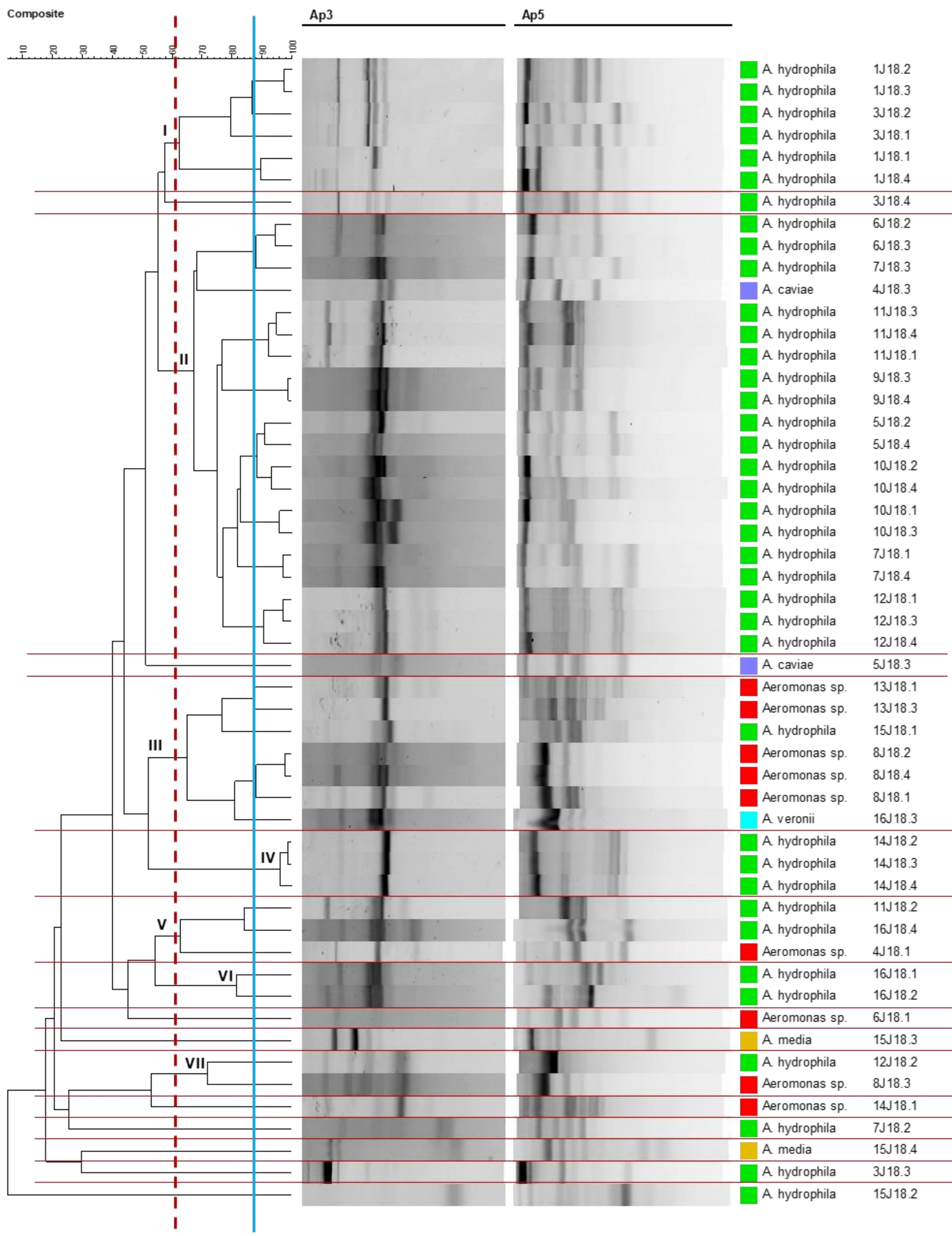
Annex IX

Supplementary Figure S8. Dendrogram of isolates collected from animals from Jamor river during the dry season based on the composite analysis of RAPD finger-prints with primers Ap3 and Ap5 (Pearson correlation coefficient and UPGMA clustering). Blue line represents reproducibility level (87.6%). Isolates displaying higher similarity levels were considered identical. Red dash line represents cluster level (60.46%). Red lines easy the visualization of the defined clusters. Cophenetic correlation coefficient was 0.87. First column represents *Aeromonas* species and second column the isolate's code.



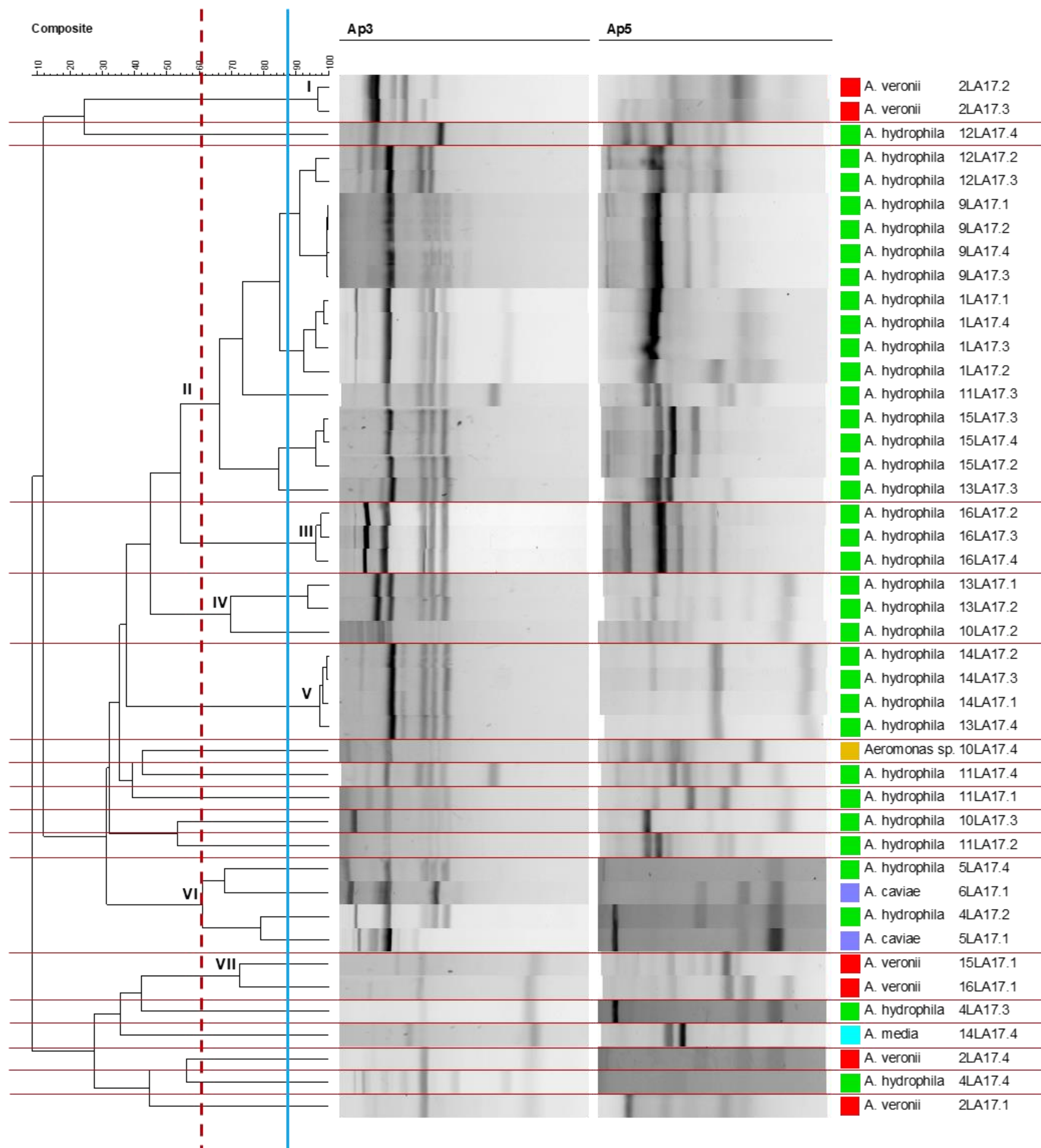
Annex X

Supplementary Figure S9. Dendrogram of isolates collected from animals from Jamor river during the wet season based on the composite analysis of RAPD finger-prints with primers Ap3 and Ap5 (Pearson correlation coefficient and UPGMA clustering). Blue line represents reproducibility level (87.6%). Isolates displaying higher similarity levels were considered identical. Red dash line represents cluster level (60.46%). Red lines easy the visualization of the defined clusters. Cophenetic correlation coefficient was 0.85. First column represents *Aeromonas* species and second column the isolate's code.



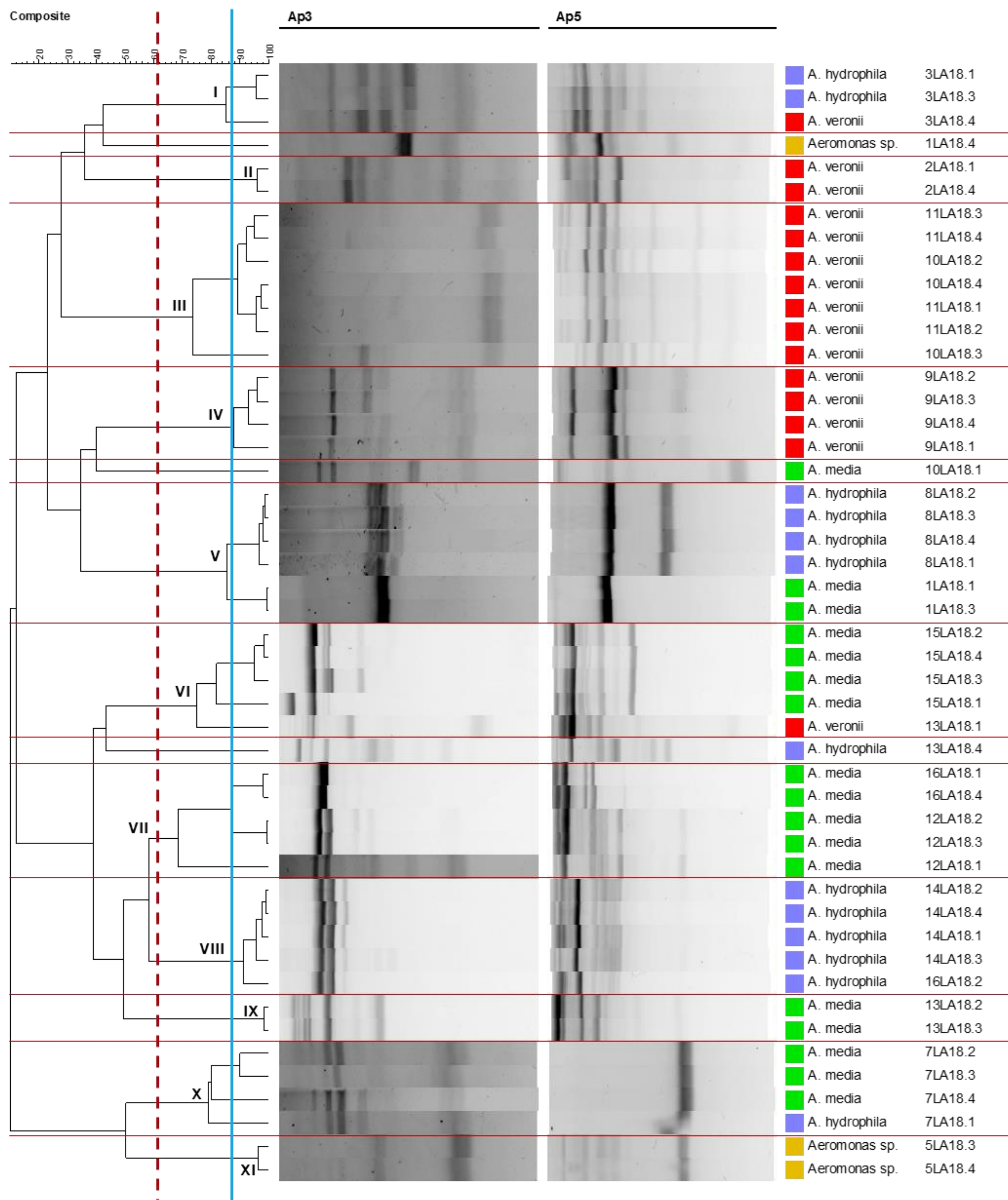
Annex XI

Supplementary Figure S10. Dendrogram of isolates collected from animals from Laje river during the dry season based on the composite analysis of RAPD finger-prints with primers Ap3 and Ap5 (Pearson correlation coefficient and UPGMA clustering). Blue line represents reproducibility level (87.6%). Isolates displaying higher similarity levels were considered identical. Red dash line represents cluster level (60.46%). Red lines easy the visualization of the defined clusters. Cophenetic correlation coefficient was 0.91. First column represents *Aeromonas* species and second column the isolate's code.



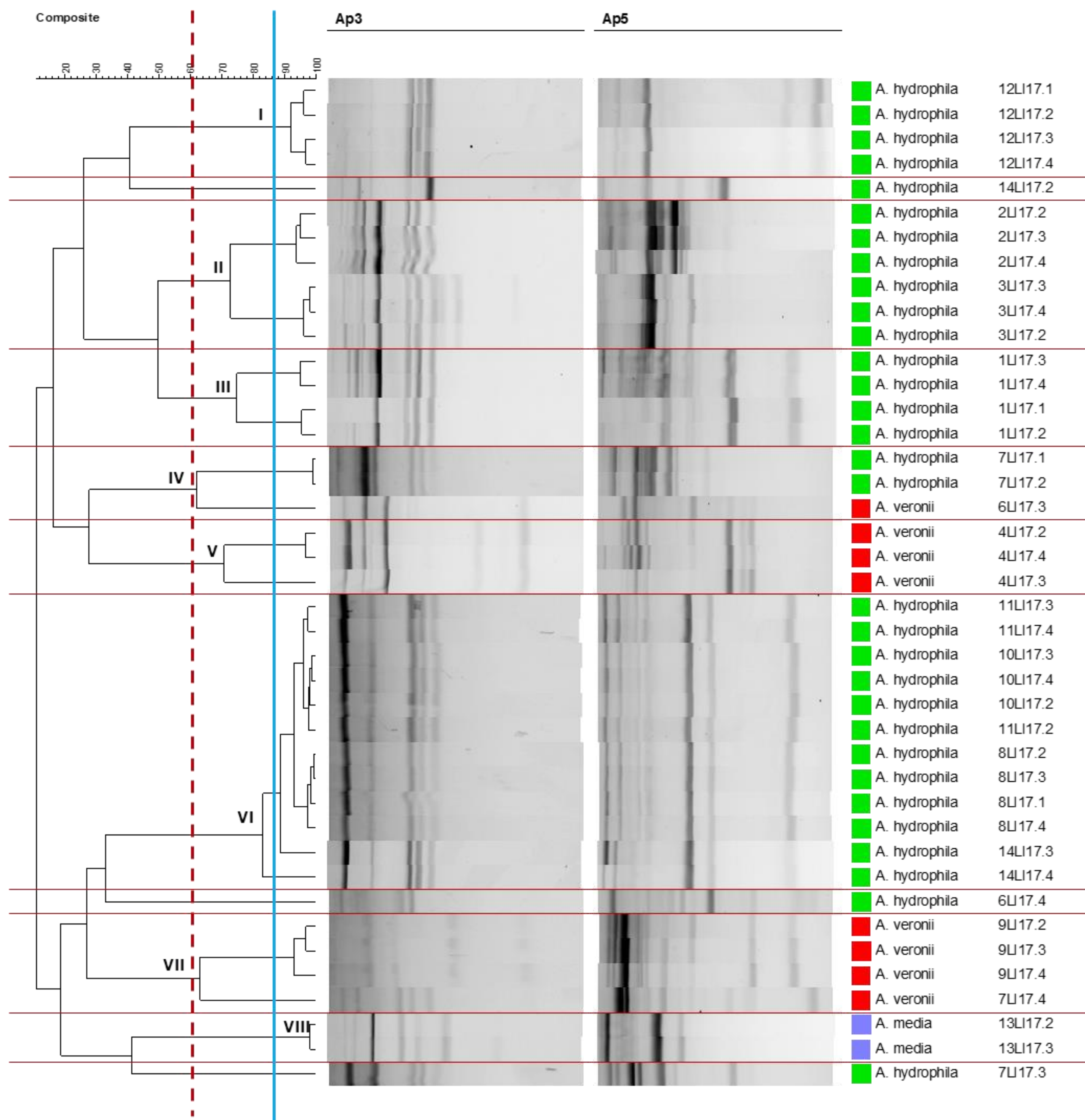
Annex XII

Supplementary Figure S11. Dendrogram of isolates collected from animals from Laje river during the wet season based on the composite analysis of RAPD finger-prints with primers Ap3 and Ap5 (Pearson correlation coefficient and UPGMA clustering). Blue line represents reproducibility level (87.6%). Isolates displaying higher similarity levels were considered identical. Red dash line represents cluster level (60.46%). Red lines easy the visualization of the defined clusters. Cophenetic correlation coefficient was 0.92. First column represents *Aeromonas* species and second column the isolate's code.



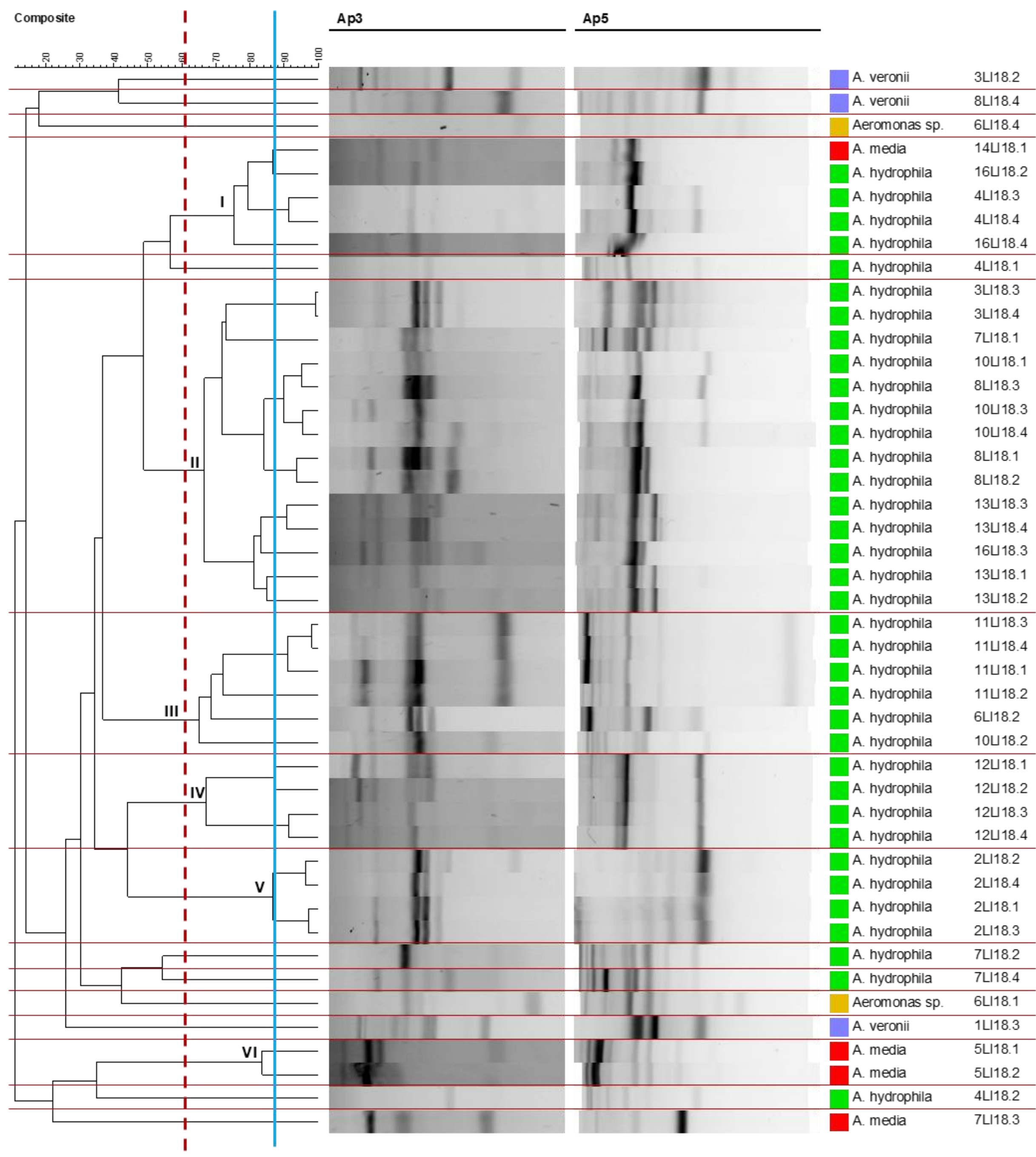
Annex XIII

Supplementary Figure S12. Dendrogram of isolates collected from animals from Lizandro river during the dry season based on the composite analysis of RAPD finger-prints with primers Ap3 and Ap5 (Pearson correlation coefficient and UPGMA clustering). Blue line represents reproducibility level (87.6%). Isolates displaying higher similarity levels were considered identical. Red dash line represents cluster level (60.46%). Red lines easy the visualization of the defined clusters. Cophenetic correlation coefficient was 0.93. First column represents *Aeromonas* species and second column the isolate's code.



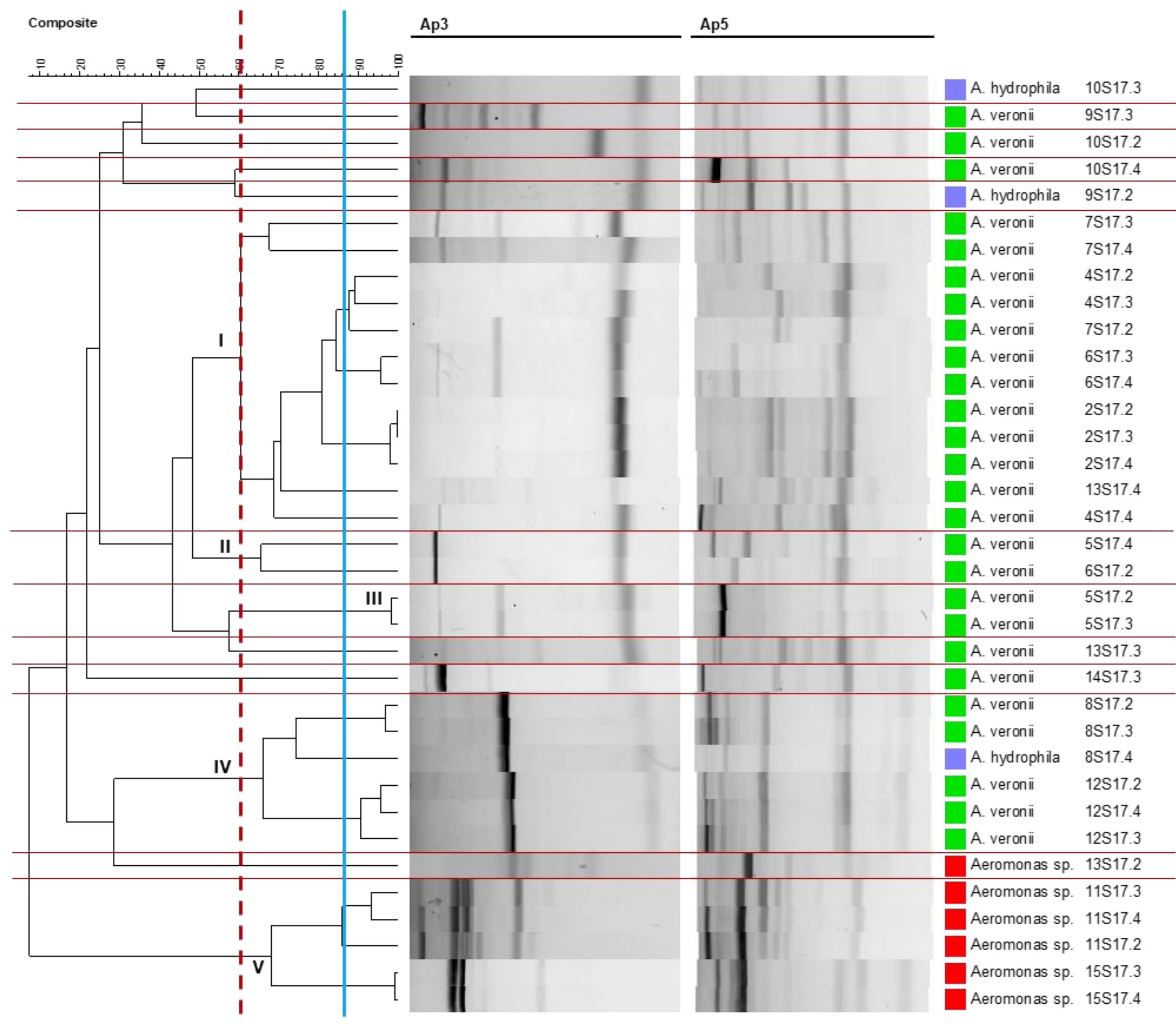
Annex XIV

Supplementary Figure S13. Dendrogram of isolates collected from animals from Lizandro river during the wet season based on the composite analysis of RAPD finger-prints with primers Ap3 and Ap5 (Pearson correlation coefficient and UPGMA clustering). Blue line represents reproducibility level (87.6%). Isolates displaying higher similarity levels were considered identical. Red dash line represents cluster level (60.46%). Red lines easy the visualization of the defined clusters. Cophenetic correlation coefficient was 0.85. First column represents *Aeromonas* species and second column the isolate's code.



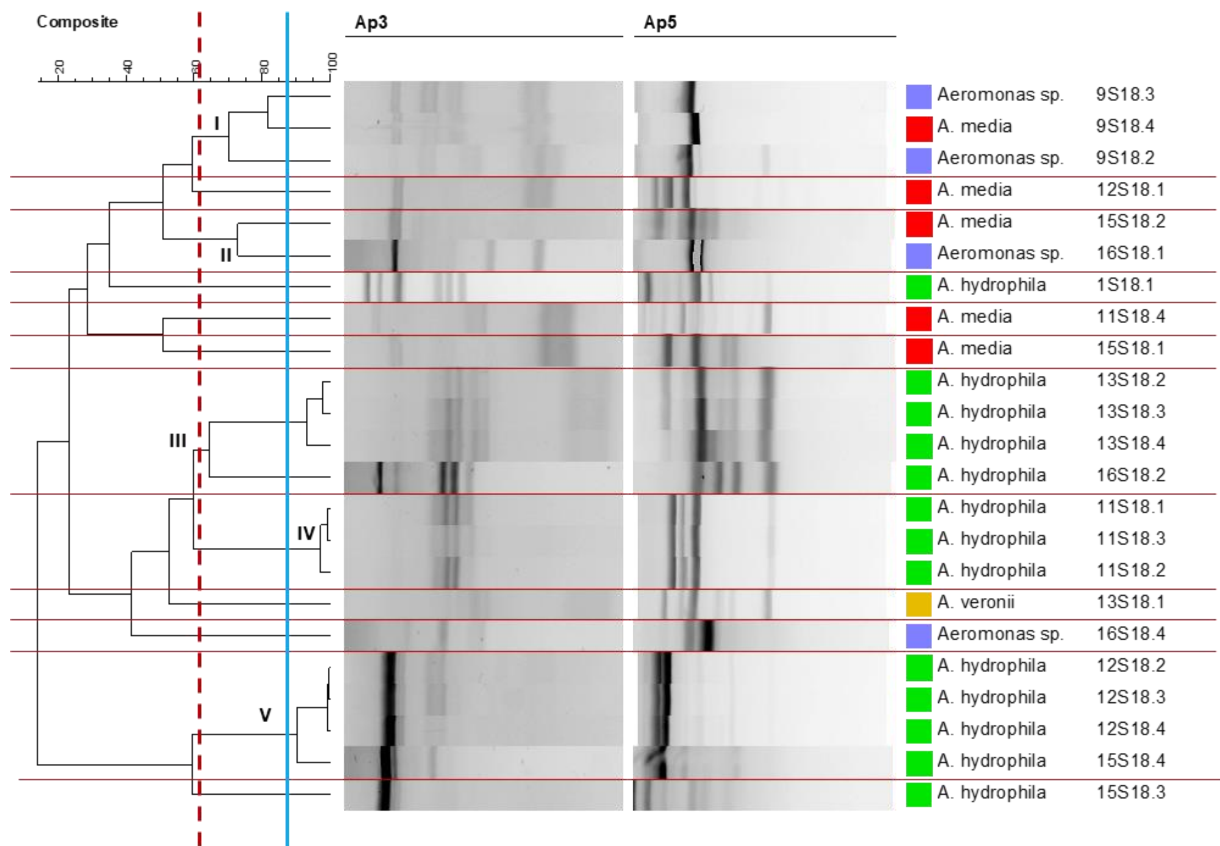
Annex XV

Supplementary Figure S14. Dendrogram of isolates collected from animals from Samarra river during the dry season based on the composite analysis of RAPD finger-prints with primers Ap3 and Ap5 (Pearson correlation coefficient and UPGMA clustering). Blue line represents reproducibility level (87.6%). Isolates displaying higher similarity levels were considered identical. Red dash line represents cluster level (60.46%). Red lines easy the visualization of the defined clusters. Cophenetic correlation coefficient was 0.92. First column represents *Aeromonas* species and second column the isolate's code.



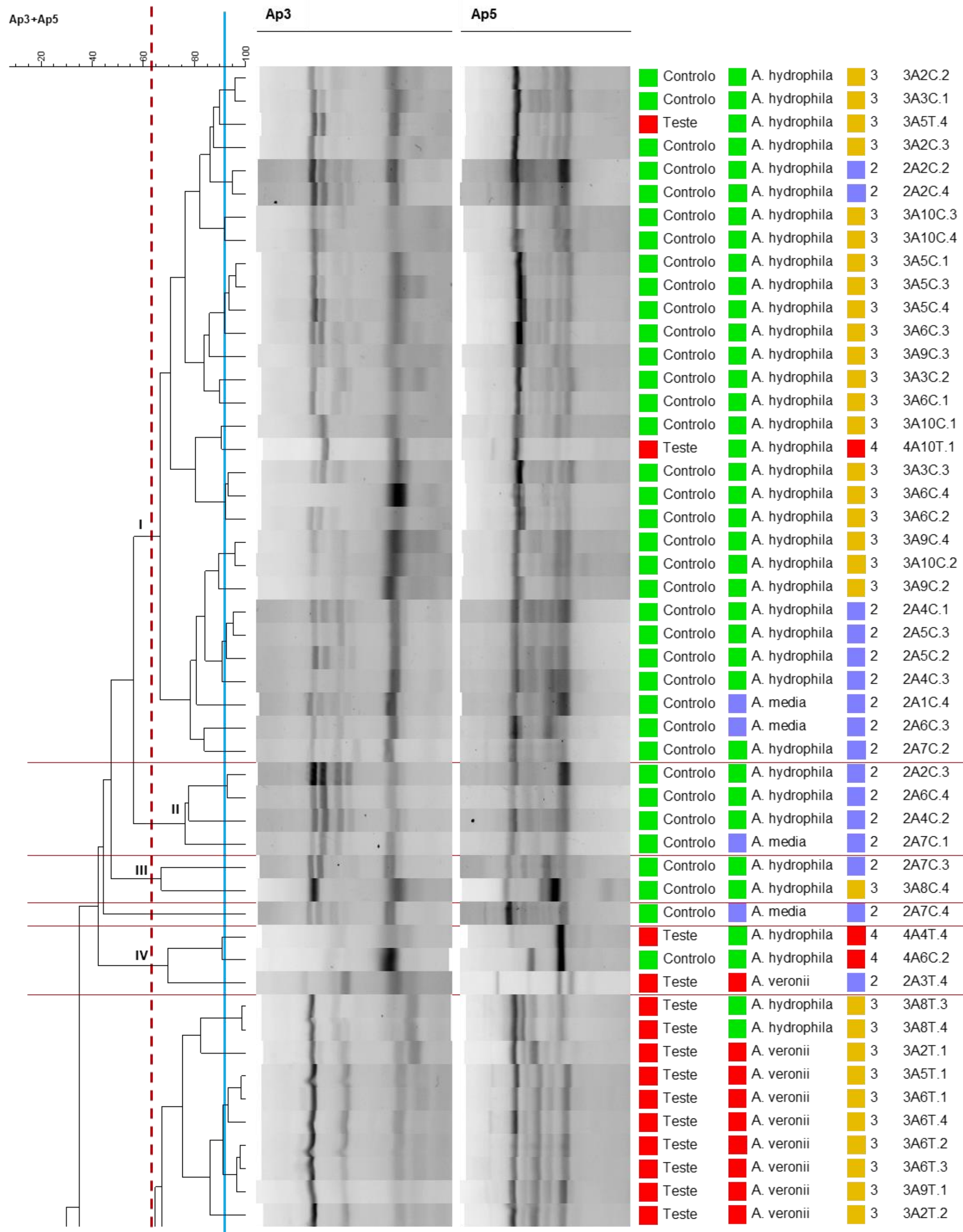
Annex XVI

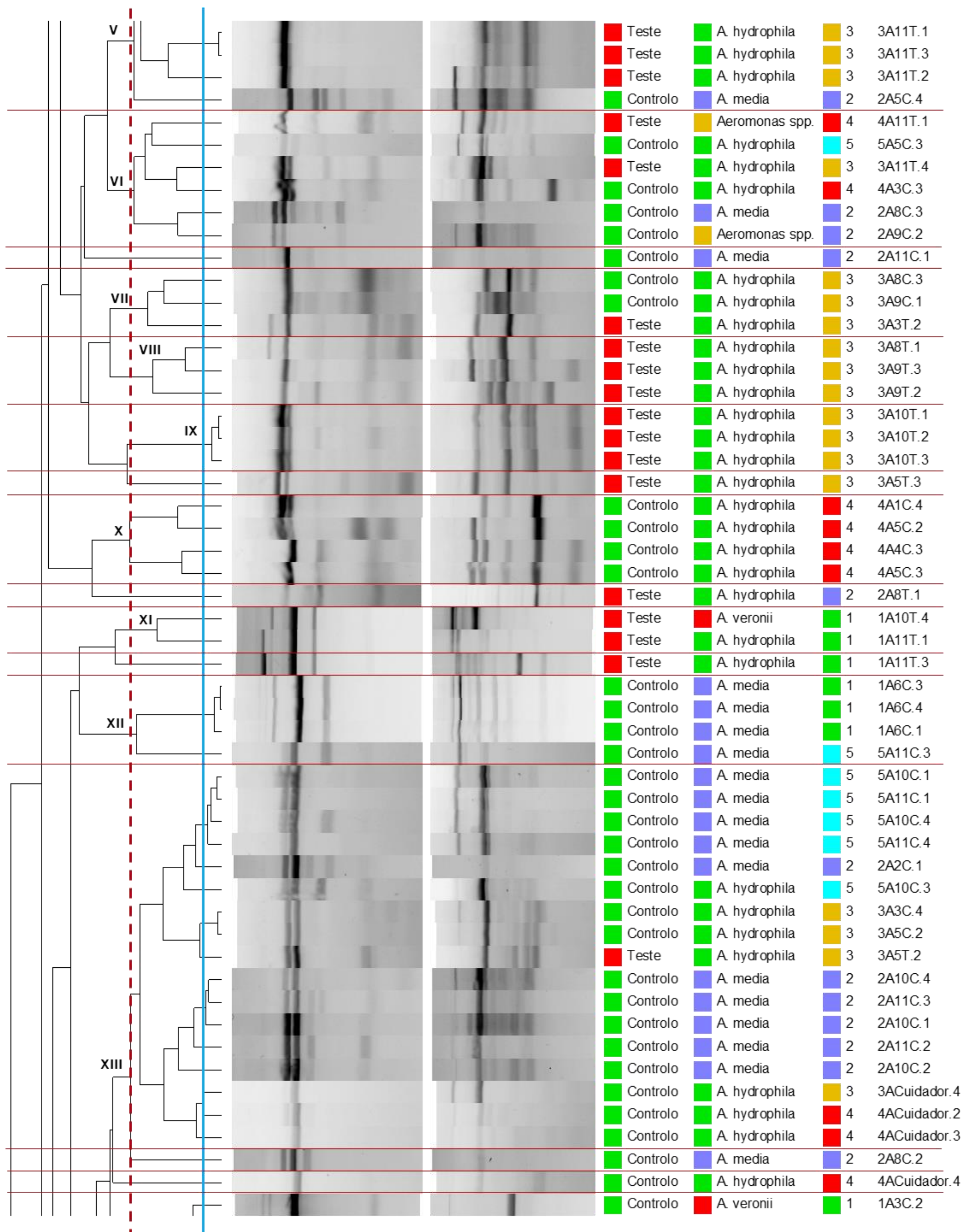
Supplementary Figure S15. Dendrogram of isolates collected from animals from Samarra river during the wet season based on the composite analysis of RAPD finger-prints with primers Ap3 and Ap5 (Pearson correlation coefficient and UPGMA clustering). Blue line represents reproducibility level (87.6%). Isolates displaying higher similarity levels were considered identical. Red dash line represents cluster level (60.46%). Red lines easy the visualization of the defined clusters. Cophenetic correlation coefficient was 0.87. First column represents *Aeromonas* species and second column the isolate's code.



Annex XVII

Supplementary Figure S16. Dendrogram based on the composite analysis of RAPD fingerprints with primers Ap3 and Ap5 (Pearson correlation coefficient and UPGMA clustering) of the entire bacterial collection. Blue line represents reproducibility level (91.88 %). Isolates displaying higher similarity levels were considered identical. Red dash line represents cut off level (62.93 %). Red lines represent cluster division. Cophenetic correlation coefficient was 0.75. First column represents the tank, second column the *Aeromonas* species, third column the sampling week and the fourth column the isolate's code. Controlo – Control, Teste – Test, Alimento – Food, Cuidador – Aquarist.

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VI
VII
VIII
IX
X
XI
XII
XIII
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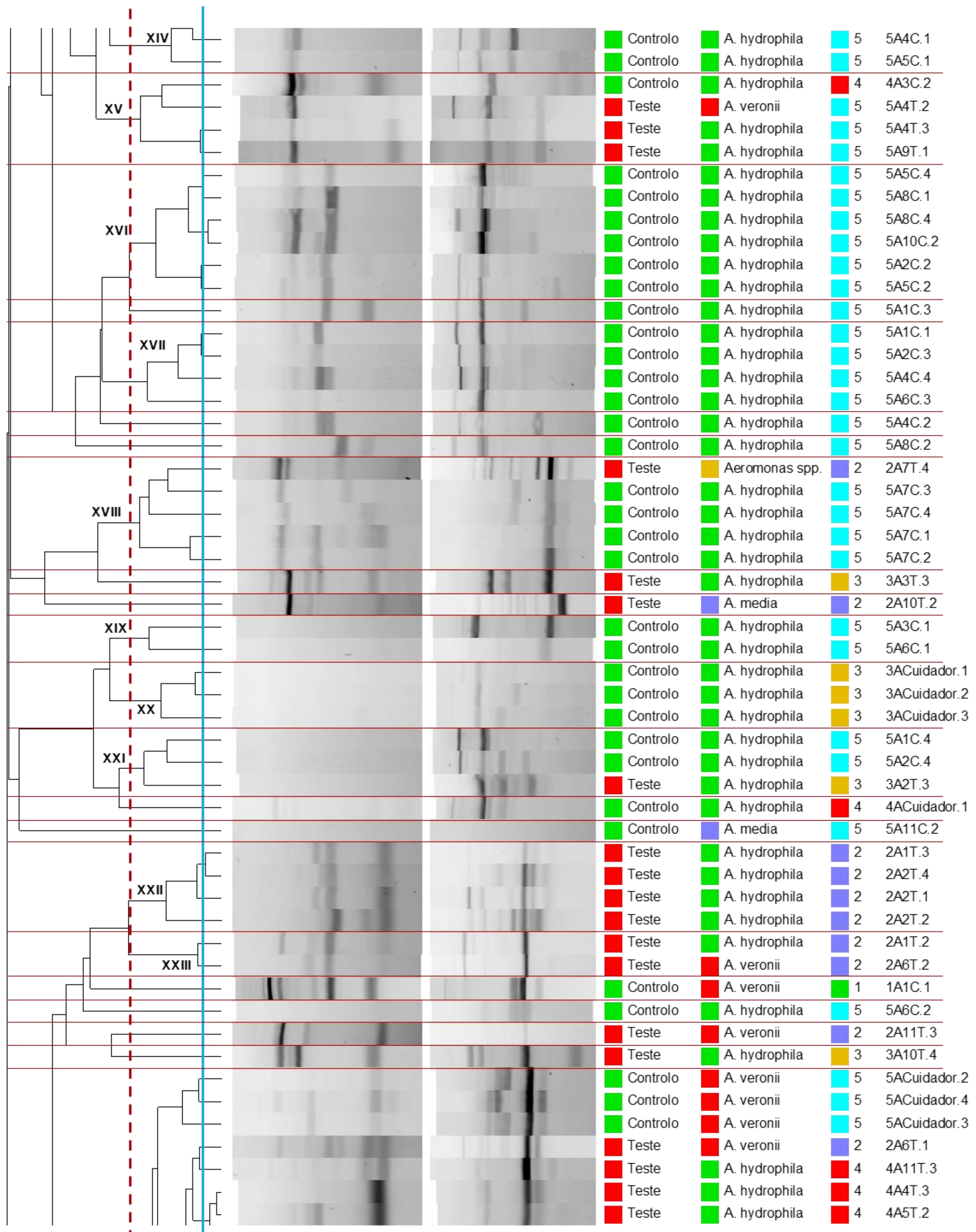
Teste A. hydrophila 3 3A11T.2
Controlo A. media 2 2A5C.4
Teste Aeromonas spp. 4 4A11T.1
Controlo A. hydrophila 5 5A5C.3
Teste A. hydrophila 3 3A11T.4
Controlo A. hydrophila 4 4A3C.3
Controlo A. media 2 2A8C.3
Controlo Aeromonas spp. 2 2A9C.2
Controlo A. media 2 2A11C.1
Controlo A. hydrophila 3 3A8C.3
Controlo A. hydrophila 3 3A9C.1
Teste A. hydrophila 3 3A3T.2
Teste A. hydrophila 3 3A8T.1
Teste A. hydrophila 3 3A9T.3
Teste A. hydrophila 3 3A9T.2
Teste A. hydrophila 3 3A10T.1
Teste A. hydrophila 3 3A10T.2
Teste A. hydrophila 3 3A10T.3
Teste A. hydrophila 3 3A5T.3
Controlo A. hydrophila 4 4A1C.4
Controlo A. hydrophila 4 4A5C.2
Controlo A. hydrophila 4 4A4C.3
Controlo A. hydrophila 4 4A5C.3
Teste A. hydrophila 2 2A8T.1
Teste A. veronii 1 1A10T.4
Teste A. hydrophila 1 1A11T.1
Teste A. hydrophila 1 1A11T.3
Controlo A. media 1 1A6C.3
Controlo A. media 1 1A6C.4
Controlo A. media 1 1A6C.1
Controlo A. media 5 5A11C.3
Controlo A. media 5 5A10C.1
Controlo A. media 5 5A11C.1
Controlo A. media 5 5A10C.4
Controlo A. media 5 5A11C.4
Controlo A. media 2 2A2C.1
Controlo A. hydrophila 5 5A10C.3
Controlo A. hydrophila 3 3A3C.4
Controlo A. hydrophila 3 3A5C.2
Teste A. hydrophila 3 3A5T.2
Controlo A. media 2 2A10C.4
Controlo A. media 2 2A11C.3
Controlo A. media 2 2A10C.1
Controlo A. media 2 2A11C.2
Controlo A. media 2 2A10C.2
Controlo A. hydrophila 3 3ACuidador.4
Controlo A. hydrophila 4 4ACuidador.2
Controlo A. hydrophila 4 4ACuidador.3
Controlo A. media 2 2A8C.2
Controlo A. hydrophila 4 4ACuidador.4
Controlo A. veronii 1 1A3C.2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XV
XVI
XVII
XVIII
XIX
XX
XXI
XXII
XXIII
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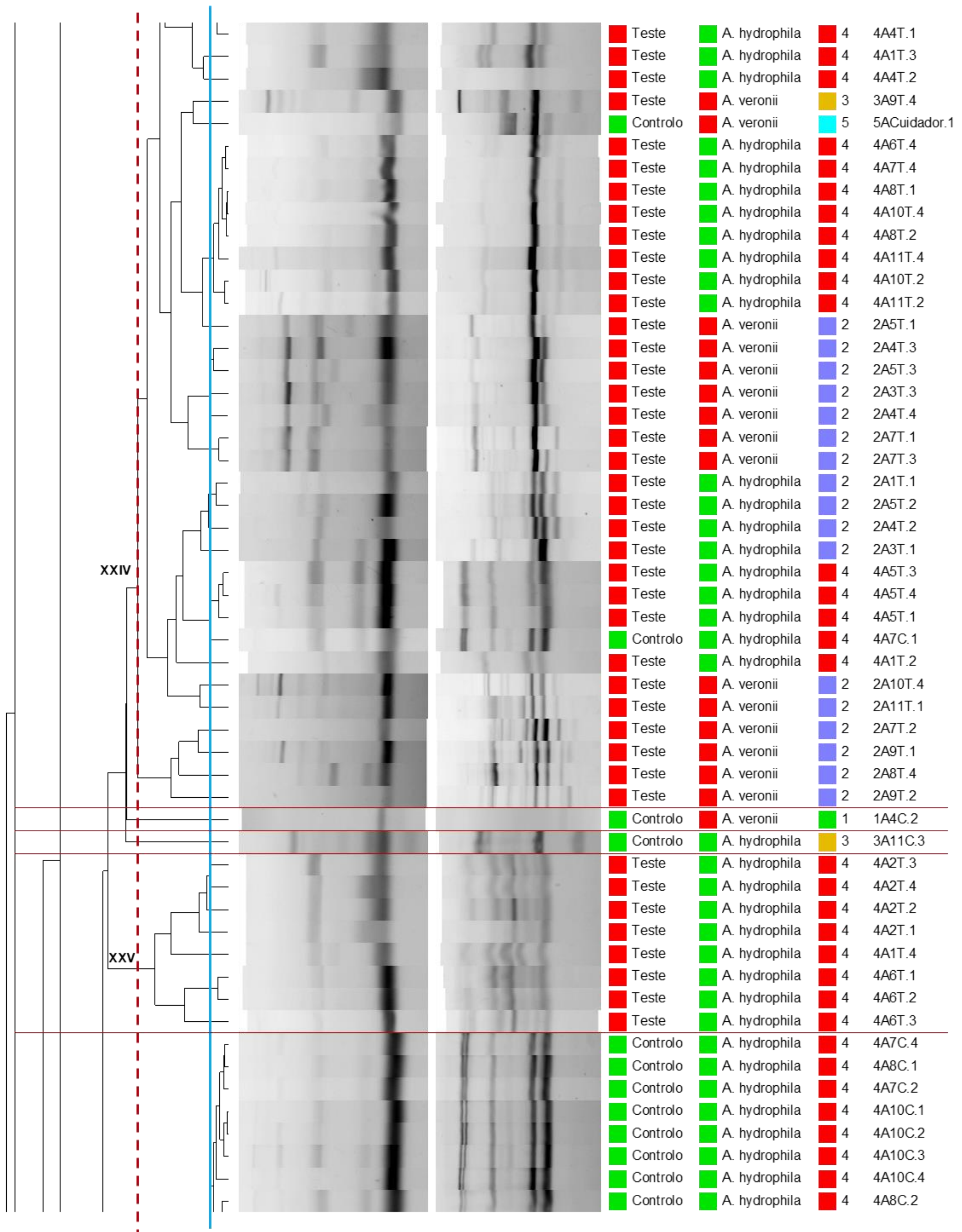
Controlo A. hydrophila 4 4A3C.2
Teste A. veronii 5 5A4T.2
Teste A. hydrophila 5 5A4T.3
Teste A. hydrophila 5 5A9T.1
Controlo A. hydrophila 5 5A5C.4
Controlo A. hydrophila 5 5A8C.1
Controlo A. hydrophila 5 5A8C.4
Controlo A. hydrophila 5 5A10C.2
Controlo A. hydrophila 5 5A2C.2
Controlo A. hydrophila 5 5A5C.2
Controlo A. hydrophila 5 5A1C.3
Controlo A. hydrophila 5 5A1C.1
Controlo A. hydrophila 5 5A2C.3
Controlo A. hydrophila 5 5A4C.4
Controlo A. hydrophila 5 5A6C.3
Controlo A. hydrophila 5 5A4C.2
Controlo A. hydrophila 5 5A8C.2
Teste Aeromonas spp. 2 2A7T.4
Controlo A. hydrophila 5 5A7C.3
Controlo A. hydrophila 5 5A7C.4
Controlo A. hydrophila 5 5A7C.1
Controlo A. hydrophila 5 5A7C.2
Teste A. hydrophila 3 3A3T.3
Teste A. media 2 2A10T.2
Controlo A. hydrophila 5 5A3C.1
Controlo A. hydrophila 5 5A6C.1
Controlo A. hydrophila 3 3ACuidador.1
Controlo A. hydrophila 3 3ACuidador.2
Controlo A. hydrophila 3 3ACuidador.3
Controlo A. hydrophila 5 5A1C.4
Controlo A. hydrophila 5 5A2C.4
Teste A. hydrophila 3 3A2T.3
Controlo A. hydrophila 4 4ACuidador.1
Controlo A. media 5 5A11C.2
Teste A. hydrophila 2 2A1T.3
Teste A. hydrophila 2 2A2T.4
Teste A. hydrophila 2 2A2T.1
Teste A. hydrophila 2 2A2T.2
Teste A. hydrophila 2 2A1T.2
Teste A. veronii 2 2A6T.2
Controlo A. veronii 1 1A1C.1
Controlo A. hydrophila 5 5A6C.2
Teste A. veronii 2 2A11T.3
Teste A. hydrophila 3 3A10T.4
Controlo A. veronii 5 5ACuidador.2
Controlo A. veronii 5 5ACuidador.4
Controlo A. veronii 5 5ACuidador.3
Teste A. veronii 2 2A6T.1
Teste A. hydrophila 4 4A11T.3
Teste A. hydrophila 4 4A4T.3
Teste A. hydrophila 4 4A5T.2



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XXV
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Teste A. hydrophila 4 4A4T.2
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Controlo A. veronii 5 5ACuidador.1
Teste A. hydrophila 4 4A6T.4
Teste A. hydrophila 4 4A7T.4
Teste A. hydrophila 4 4A8T.1
Teste A. hydrophila 4 4A10T.4
Teste A. hydrophila 4 4A8T.2
Teste A. hydrophila 4 4A11T.4
Teste A. hydrophila 4 4A10T.2
Teste A. hydrophila 4 4A11T.2
Teste A. veronii 2 2A5T.1
Teste A. veronii 2 2A4T.3
Teste A. veronii 2 2A5T.3
Teste A. veronii 2 2A3T.3
Teste A. veronii 2 2A4T.4
Teste A. veronii 2 2A7T.1
Teste A. veronii 2 2A7T.3
Teste A. hydrophila 2 2A1T.1
Teste A. hydrophila 2 2A5T.2
Teste A. hydrophila 2 2A4T.2
Teste A. hydrophila 2 2A3T.1
Teste A. hydrophila 4 4A5T.3
Teste A. hydrophila 4 4A5T.4
Teste A. hydrophila 4 4A5T.1
Controlo A. hydrophila 4 4A7C.1
Teste A. hydrophila 4 4A1T.2
Teste A. veronii 2 2A10T.4
Teste A. veronii 2 2A11T.1
Teste A. veronii 2 2A7T.2
Teste A. veronii 2 2A9T.1
Teste A. veronii 2 2A8T.4
Teste A. veronii 2 2A9T.2
Controlo A. veronii 1 1A4C.2
Controlo A. hydrophila 3 3A11C.3
Teste A. hydrophila 4 4A2T.3
Teste A. hydrophila 4 4A2T.4
Teste A. hydrophila 4 4A2T.2
Teste A. hydrophila 4 4A2T.1
Teste A. hydrophila 4 4A1T.4
Teste A. hydrophila 4 4A6T.1
Teste A. hydrophila 4 4A6T.2
Teste A. hydrophila 4 4A6T.3
Controlo A. hydrophila 4 4A7C.4
Controlo A. hydrophila 4 4A8C.1
Controlo A. hydrophila 4 4A7C.2
Controlo A. hydrophila 4 4A10C.1
Controlo A. hydrophila 4 4A10C.2
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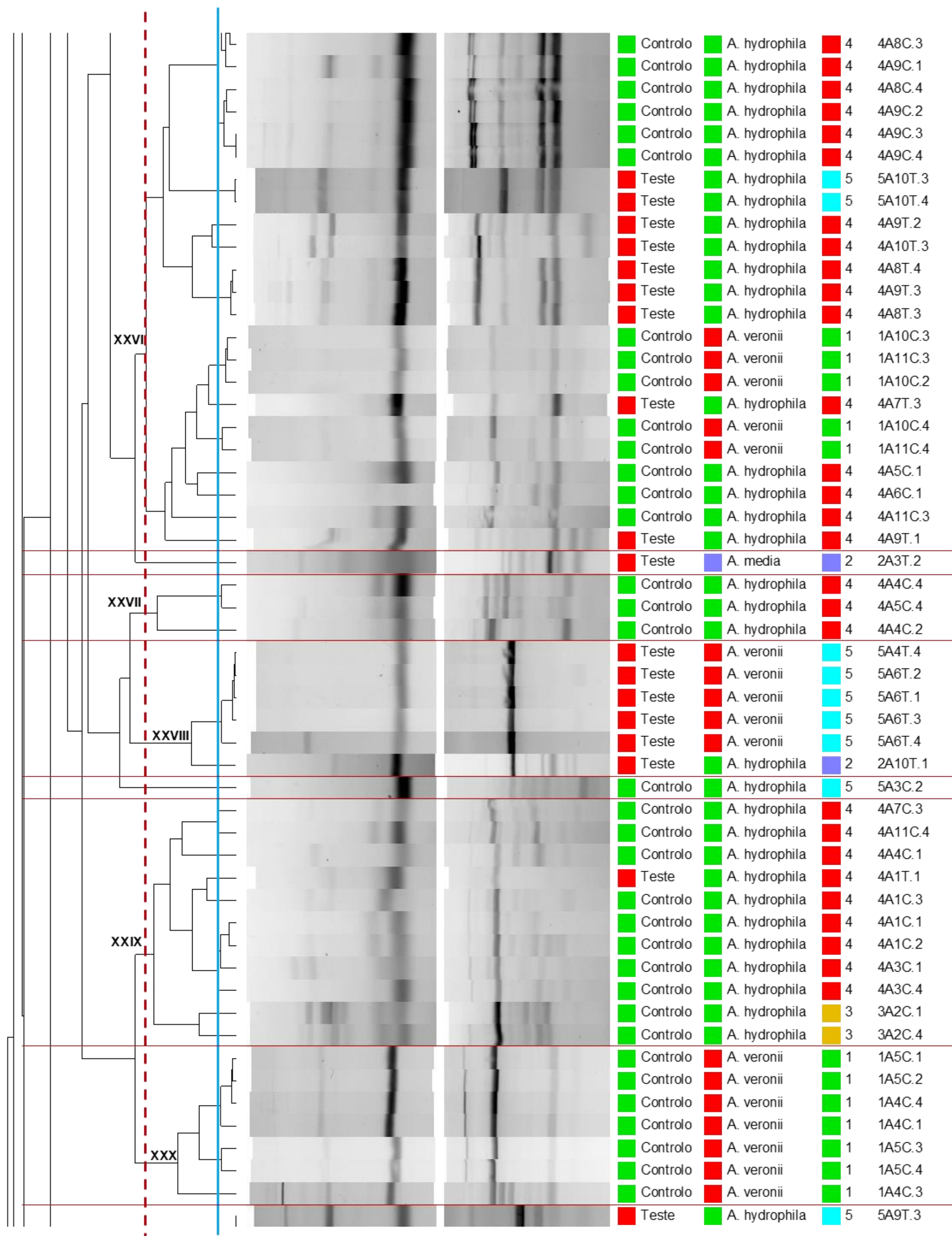
Controlo A. hydrophila 4 4A10C.4
Controlo A. hydrophila 4 4A8C.2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XXVIII
XXIX
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Controlo A. hydrophila 4 4A8C.4
Controlo A. hydrophila 4 4A9C.2
Controlo A. hydrophila 4 4A9C.3
Controlo A. hydrophila 4 4A9C.4
Teste A. hydrophila 5 5A10T.3
Teste A. hydrophila 5 5A10T.4
Teste A. hydrophila 4 4A9T.2
Teste A. hydrophila 4 4A10T.3
Teste A. hydrophila 4 4A8T.4
Teste A. hydrophila 4 4A9T.3
Teste A. hydrophila 4 4A8T.3
Controlo A. veronii 1 1A10C.3
Controlo A. veronii 1 1A11C.3
Controlo A. veronii 1 1A10C.2
Teste A. hydrophila 4 4A7T.3
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Controlo A. veronii 1 1A11C.4
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Controlo A. hydrophila 4 4A6C.1
Controlo A. hydrophila 4 4A11C.3
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Controlo A. hydrophila 4 4A4C.4
Controlo A. hydrophila 4 4A5C.4
Controlo A. hydrophila 4 4A4C.2
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Teste A. veronii 5 5A6T.2
Teste A. veronii 5 5A6T.1
Teste A. veronii 5 5A6T.3
Teste A. veronii 5 5A6T.4
Teste A. hydrophila 2 2A10T.1
Controlo A. hydrophila 5 5A3C.2
Controlo A. hydrophila 4 4A7C.3
Controlo A. hydrophila 4 4A11C.4
Controlo A. hydrophila 4 4A4C.1
Teste A. hydrophila 4 4A1T.1
Controlo A. hydrophila 4 4A1C.3
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Controlo A. hydrophila 4 4A1C.2
Controlo A. hydrophila 4 4A3C.1
Controlo A. hydrophila 4 4A3C.4
Controlo A. hydrophila 3 3A2C.1
Controlo A. hydrophila 3 3A2C.4
Controlo A. veronii 1 1A5C.1
Controlo A. veronii 1 1A5C.2
Controlo A. veronii 1 1A4C.4
Controlo A. veronii 1 1A4C.1
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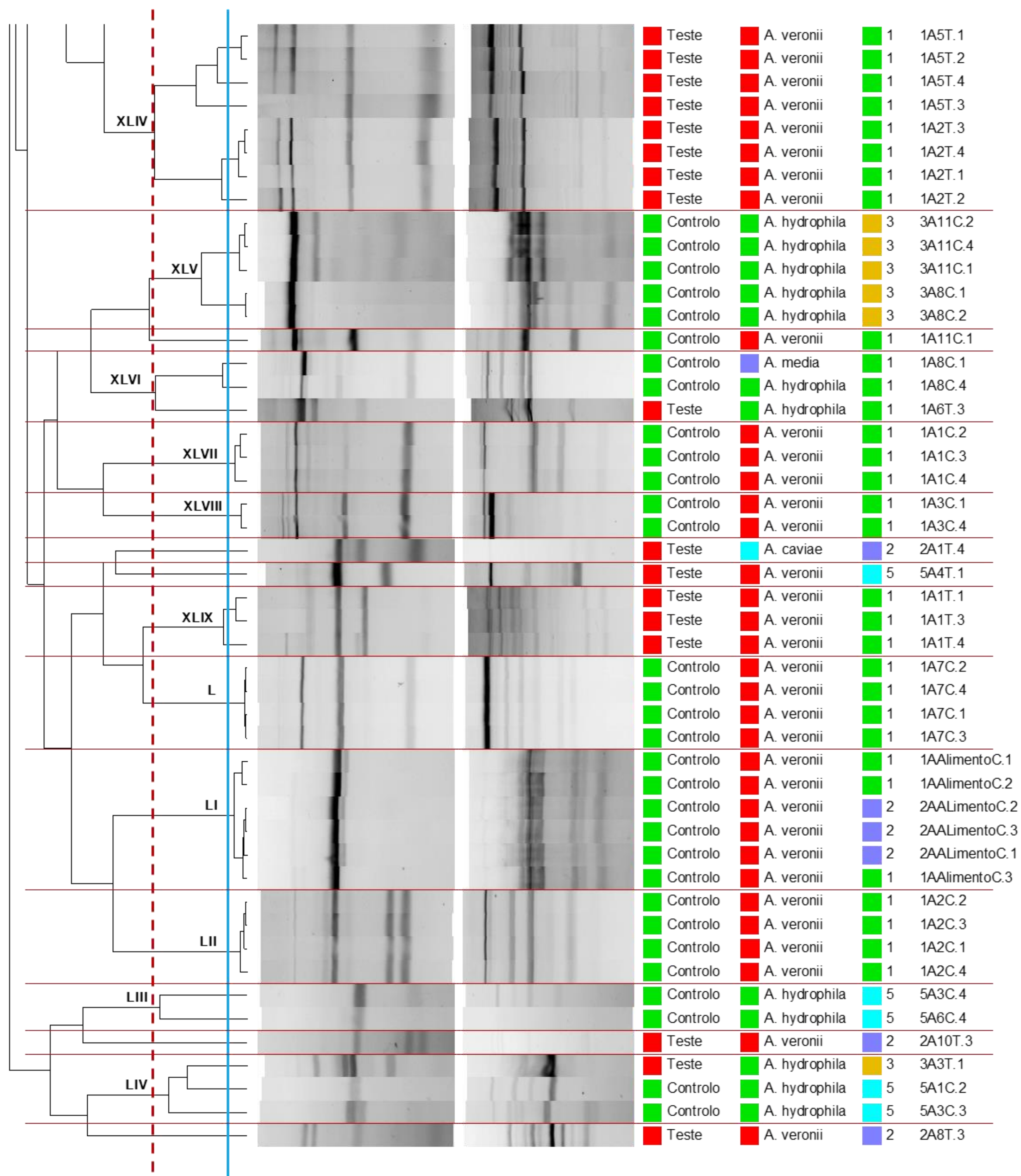

XLIV
XLV
XLVI
XLVII
XLVIII
XLIX
L
LI
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LIII
LIV
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Teste A. veronii 1 1A5T.2
Teste A. veronii 1 1A5T.4
Teste A. veronii 1 1A5T.3
Teste A. veronii 1 1A2T.3
Teste A. veronii 1 1A2T.4
Teste A. veronii 1 1A2T.1
Teste A. veronii 1 1A2T.2
Controlo A. hydrophila 3 3A11C.2
Controlo A. hydrophila 3 3A11C.4
Controlo A. hydrophila 3 3A11C.1
Controlo A. hydrophila 3 3A8C.1
Controlo A. hydrophila 3 3A8C.2
Controlo A. veronii 1 1A11C.1
Controlo A. media 1 1A8C.1
Controlo A. hydrophila 1 1A8C.4
Teste A. hydrophila 1 1A6T.3
Controlo A. veronii 1 1A1C.2
Controlo A. veronii 1 1A1C.3
Controlo A. veronii 1 1A1C.4
Controlo A. veronii 1 1A3C.1
Controlo A. veronii 1 1A3C.4
Teste A. caviae 2 2A1T.4
Teste A. veronii 5 5A4T.1
Teste A. veronii 1 1A1T.1
Teste A. veronii 1 1A1T.3
Teste A. veronii 1 1A1T.4
Controlo A. veronii 1 1A7C.2
Controlo A. veronii 1 1A7C.4
Controlo A. veronii 1 1A7C.1
Controlo A. veronii 1 1A7C.3
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Controlo A. veronii 2 2AALimentoC.3
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Controlo A. veronii 1 1A2C.2
Controlo A. veronii 1 1A2C.3
Controlo A. veronii 1 1A2C.1
Controlo A. veronii 1 1A2C.4
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Controlo A. hydrophila 5 5A6C.4
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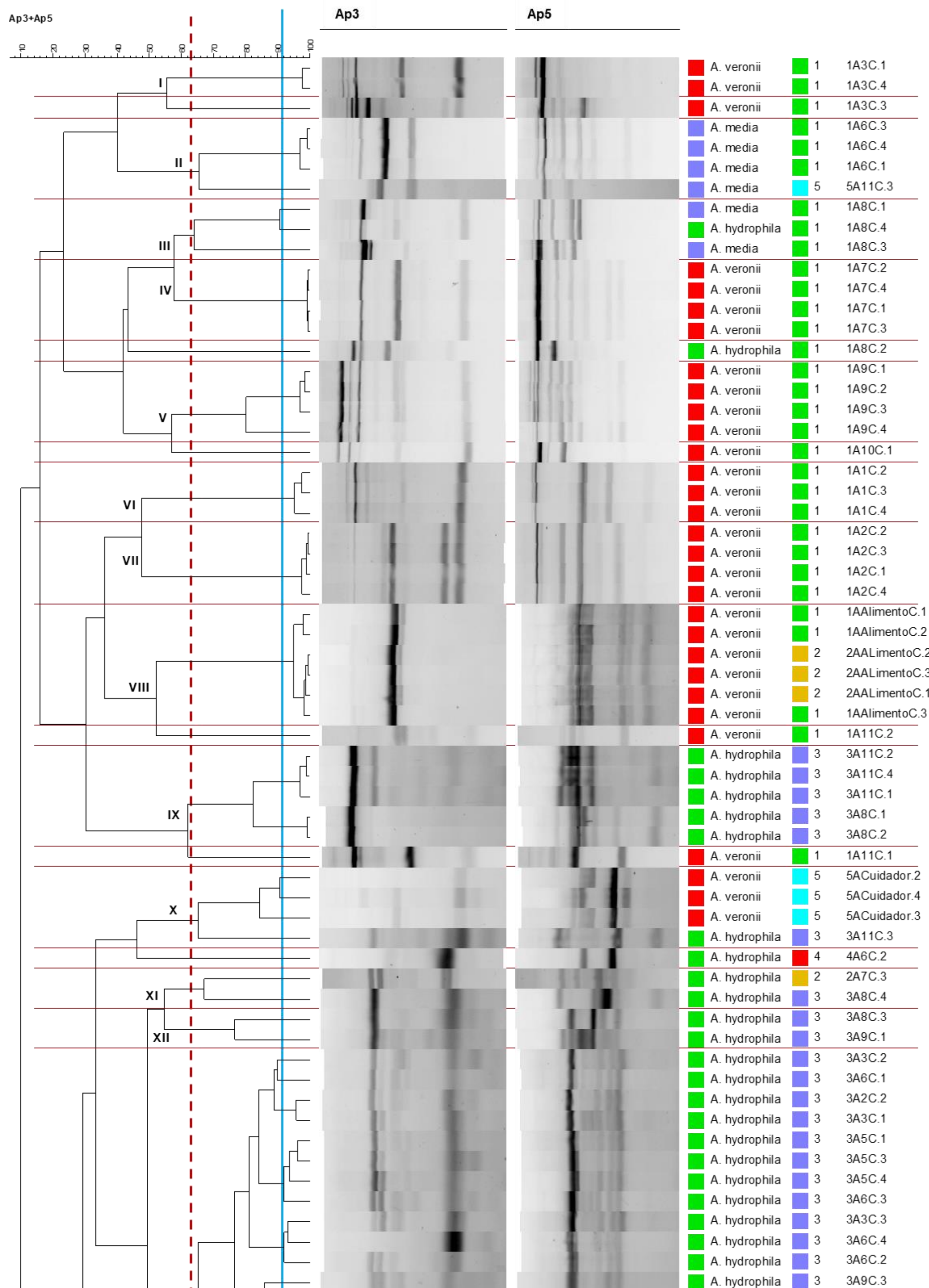
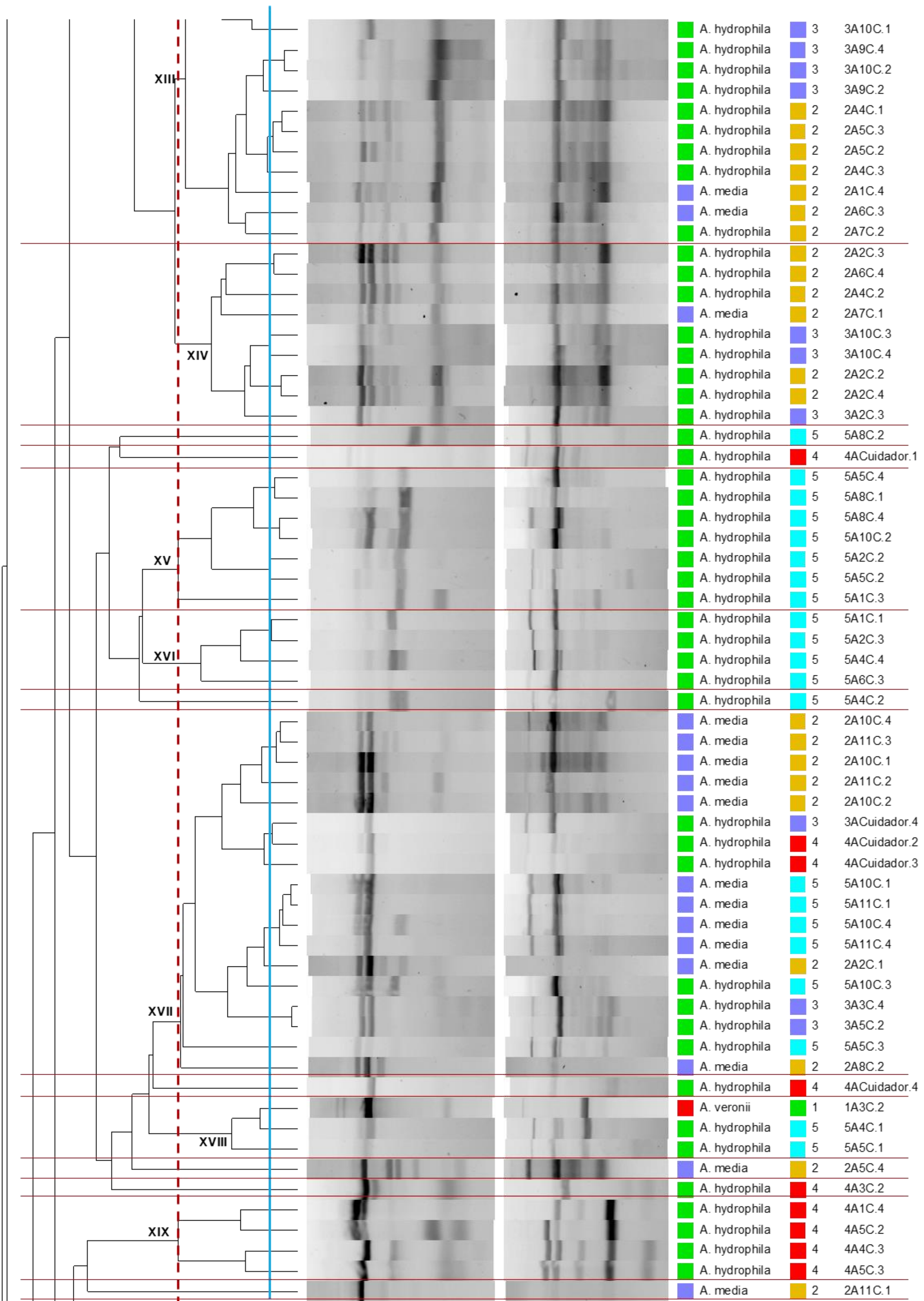
Teste A. hydrophila 3 3A3T.1
Controlo A. hydrophila 5 5A1C.2
Controlo A. hydrophila 5 5A3C.3
Teste A. veronii 2 2A8T.3

Annex XVIII

Supplementary Figure S17. Dendrograms of the bacterial collection from the control tank based on the composite analysis of RAPD fingerprints with primers Ap3 and Ap5 (Pearson correlation coefficient and UPGMA clustering). Blue line represents reproducibility level (91.88 %). Isolates displaying higher similarity levels were considered identical. Red dash line represents cut off level (62.93 %). Red lines represent cluster division. Cophenetic correlation coefficient was 0.78 for the control tank and 0.85 for the test tank. First column represents the *Aeromonas* species, second column the sampling week and the third column the isolate's code. Alimento – Food, Cuidador – Aquarist.




XIII
XIV
XV
XVI
XVII
XVIII
XIX
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A. hydrophila 3 3A10C.2
A. hydrophila 3 3A9C.2
A. hydrophila 2 2A4C.1
A. hydrophila 2 2A5C.3
A. hydrophila 2 2A5C.2
A. hydrophila 2 2A4C.3
A. media 2 2A1C.4
A. media 2 2A6C.3
A. hydrophila 2 2A7C.2
A. hydrophila 2 2A2C.3
A. hydrophila 2 2A6C.4
A. hydrophila 2 2A4C.2
A. media 2 2A7C.1
A. hydrophila 3 3A10C.3
A. hydrophila 3 3A10C.4
A. hydrophila 2 2A2C.2
A. hydrophila 2 2A2C.4
A. hydrophila 3 3A2C.3
A. hydrophila 5 5A8C.2
A. hydrophila 4 4ACuidador.1
A. hydrophila 5 5A5C.4
A. hydrophila 5 5A8C.1
A. hydrophila 5 5A8C.4
A. hydrophila 5 5A10C.2
A. hydrophila 5 5A2C.2
A. hydrophila 5 5A5C.2
A. hydrophila 5 5A1C.3
A. hydrophila 5 5A1C.1
A. hydrophila 5 5A2C.3
A. hydrophila 5 5A4C.4
A. hydrophila 5 5A6C.3
A. hydrophila 5 5A4C.2
A. media 2 2A10C.4
A. media 2 2A11C.3
A. media 2 2A10C.1
A. media 2 2A11C.2
A. media 2 2A10C.2
A. hydrophila 3 3ACuidador.4
A. hydrophila 4 4ACuidador.2
A. hydrophila 4 4ACuidador.3
A. media 5 5A10C.1
A. media 5 5A11C.1
A. media 5 5A10C.4
A. media 5 5A11C.4
A. media 2 2A2C.1
A. hydrophila 5 5A10C.3
A. hydrophila 3 3A3C.4
A. hydrophila 3 3A5C.2
A. hydrophila 5 5A5C.3
A. media 2 2A8C.2
A. hydrophila 4 4ACuidador.4
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A. hydrophila 5 5A4C.1
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A. hydrophila 4 4A3C.2
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A. hydrophila 4 4A4C.3
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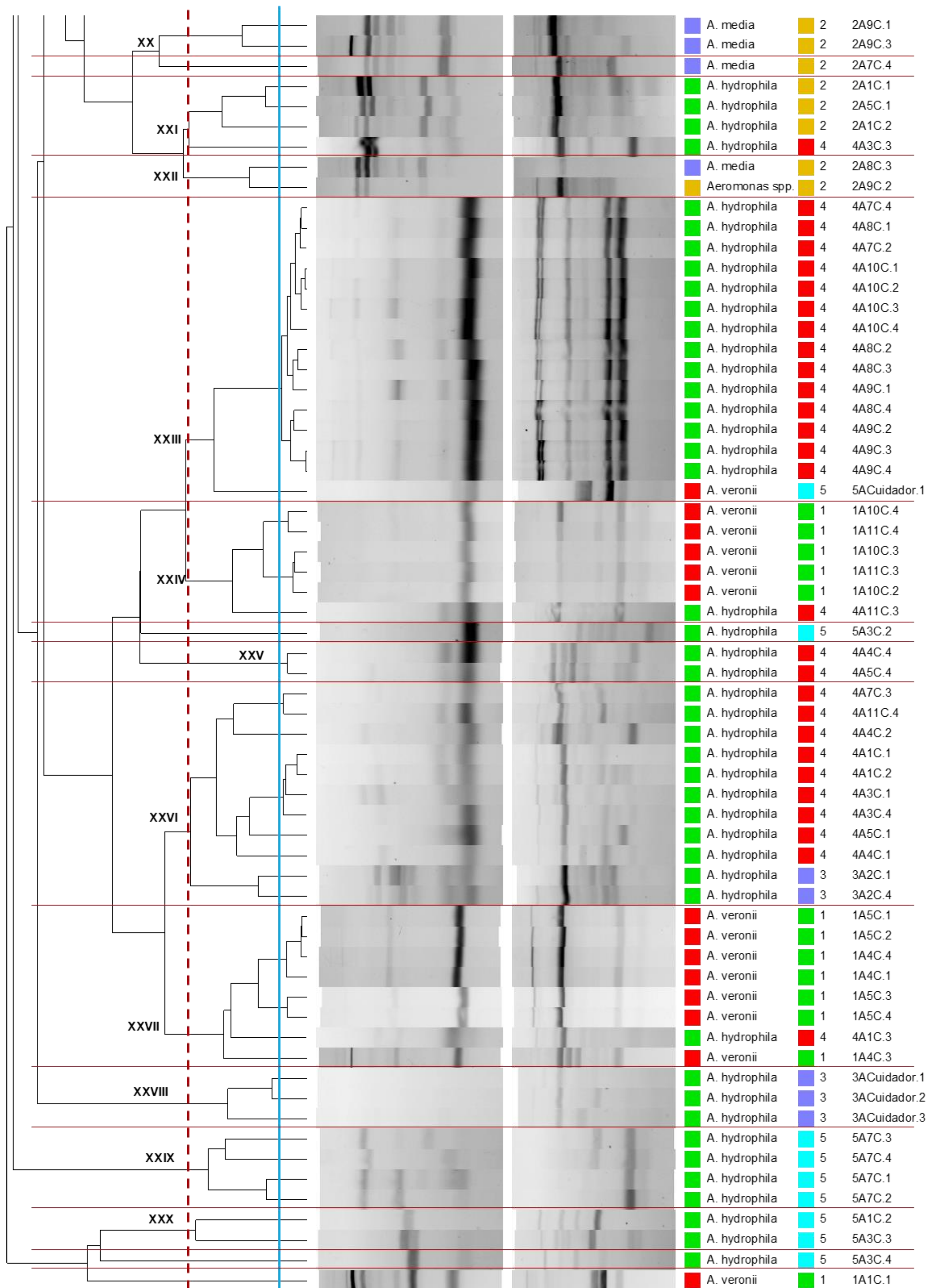
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XXIV
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XXVI
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XXVIII
XXIX
XXX
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A. media 2 2A7C.4
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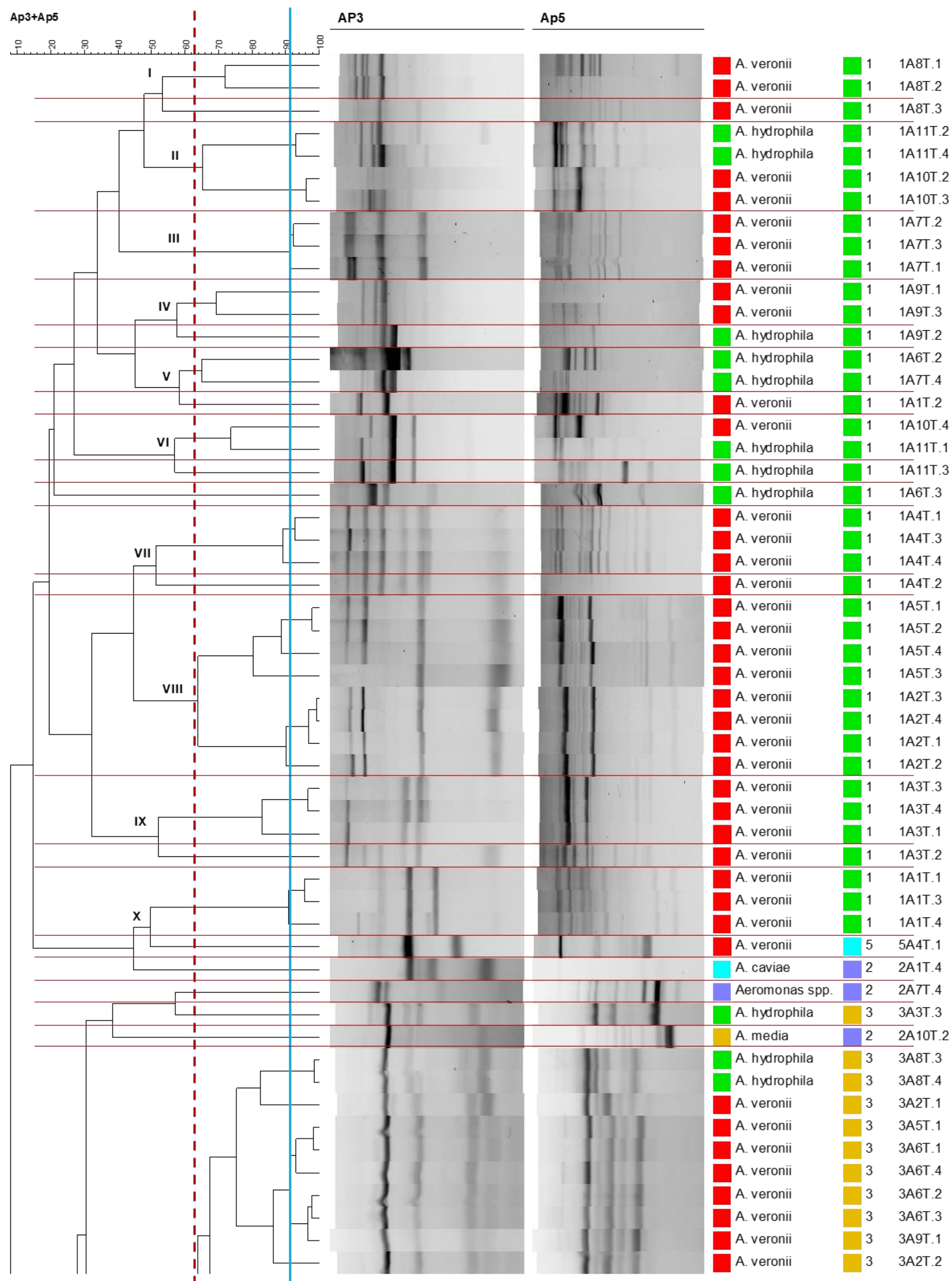
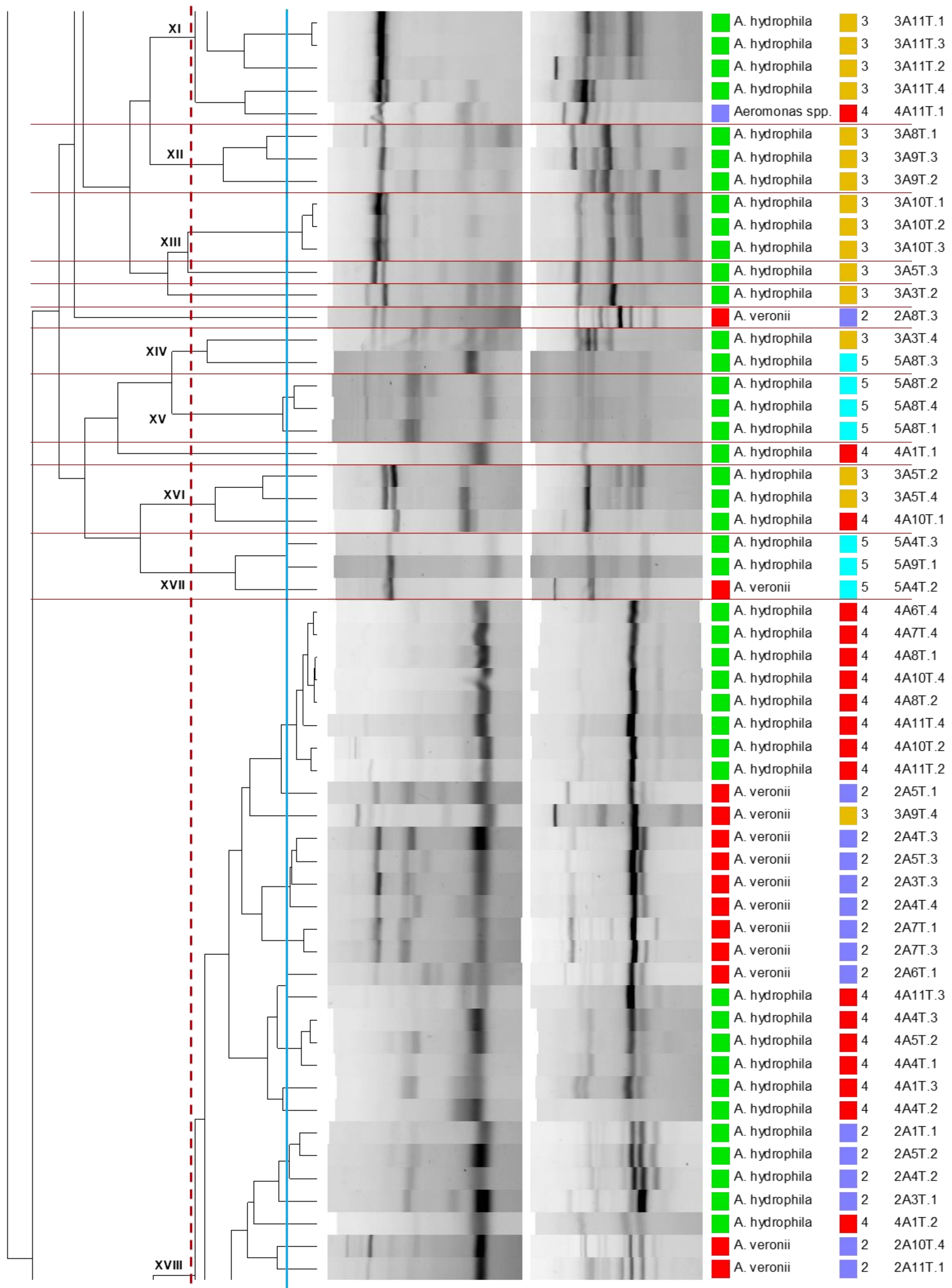
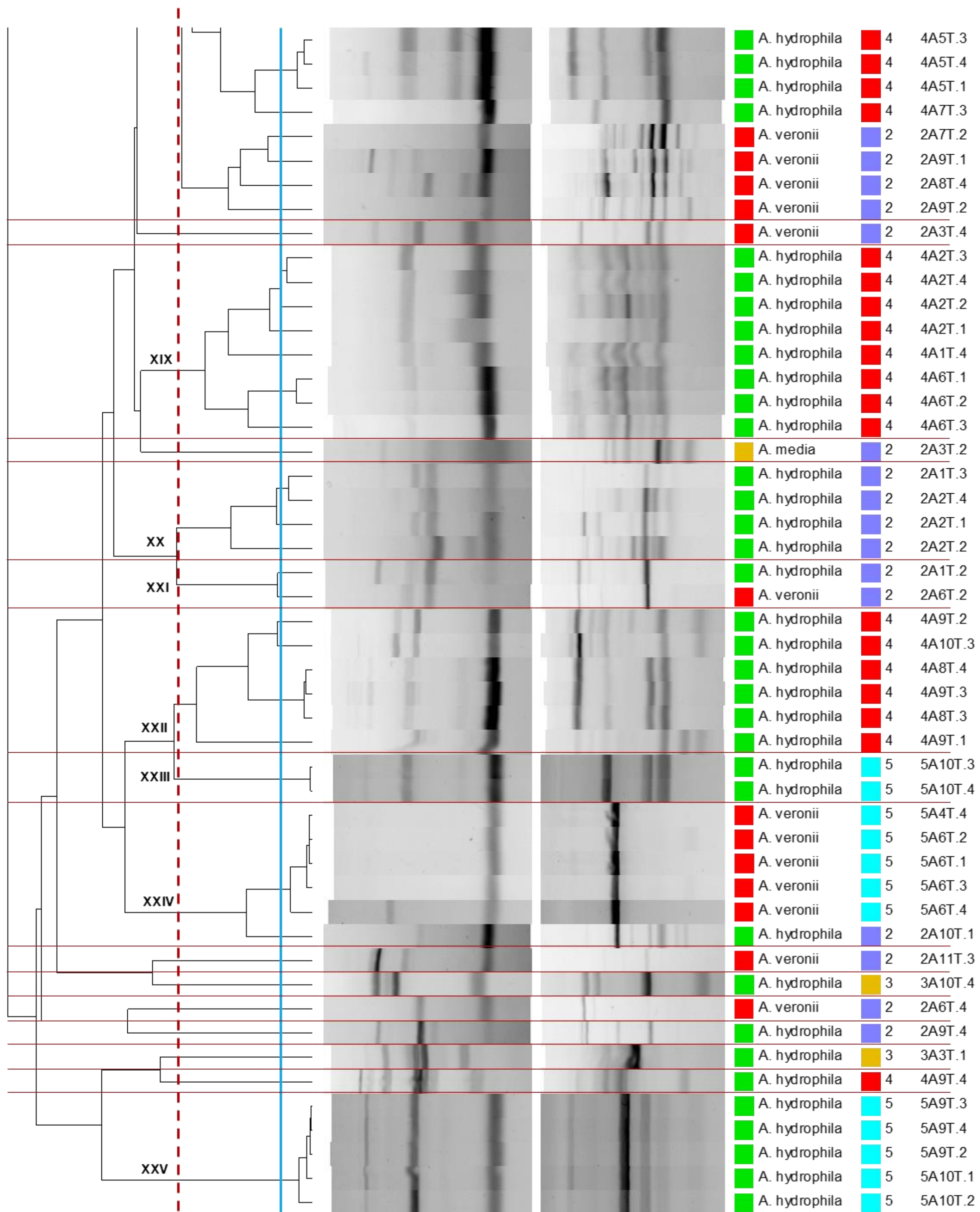
A. hydrophila 2 2A5C.1
A. hydrophila 2 2A1C.2
A. hydrophila 4 4A3C.3
A. media 2 2A8C.3
Aeromonas spp. 2 2A9C.2
A. hydrophila 4 4A7C.4
A. hydrophila 4 4A8C.1
A. hydrophila 4 4A7C.2
A. hydrophila 4 4A10C.1
A. hydrophila 4 4A10C.2
A. hydrophila 4 4A10C.3
A. hydrophila 4 4A10C.4
A. hydrophila 4 4A8C.2
A. hydrophila 4 4A8C.3
A. hydrophila 4 4A9C.1
A. hydrophila 4 4A8C.4
A. hydrophila 4 4A9C.2
A. hydrophila 4 4A9C.3
A. hydrophila 4 4A9C.4
A. veronii 5 5ACuidador.1
A. veronii 1 1A10C.4
A. veronii 1 1A11C.4
A. veronii 1 1A10C.3
A. veronii 1 1A11C.3
A. veronii 1 1A10C.2
A. hydrophila 4 4A11C.3
A. hydrophila 5 5A3C.2
A. hydrophila 4 4A4C.4
A. hydrophila 4 4A5C.4
A. hydrophila 4 4A7C.3
A. hydrophila 4 4A11C.4
A. hydrophila 4 4A4C.2
A. hydrophila 4 4A1C.1
A. hydrophila 4 4A1C.2
A. hydrophila 4 4A3C.1
A. hydrophila 4 4A3C.4
A. hydrophila 4 4A5C.1
A. hydrophila 4 4A4C.1
A. hydrophila 3 3A2C.1
A. hydrophila 3 3A2C.4
A. veronii 1 1A5C.1
A. veronii 1 1A5C.2
A. veronii 1 1A4C.4
A. veronii 1 1A4C.1
A. veronii 1 1A5C.3
A. veronii 1 1A5C.4
A. hydrophila 4 4A1C.3
A. veronii 1 1A4C.3
A. hydrophila 3 3ACuidador.1
A. hydrophila 3 3ACuidador.2
A. hydrophila 3 3ACuidador.3
A. hydrophila 5 5A7C.3
A. hydrophila 5 5A7C.4
A. hydrophila 5 5A7C.1
A. hydrophila 5 5A7C.2
A. hydrophila 5 5A1C.2
A. hydrophila 5 5A3C.3
A. hydrophila 5 5A3C.4
A. veronii 1 1A1C.1

Annex XIX

Supplementary Figure S18. Dendrograms of the bacterial collection from the test tank based on the composite analysis of RAPD fingerprints with primers Ap3 and Ap5 (Pearson correlation coefficient and UPGMA clustering). Blue line represents reproducibility level (91.88 %). Isolates displaying higher similarity levels were considered identical. Red dash line represents cut off level (62.93 %). Red lines represent cluster division. Cophenetic correlation coefficient was 0.78 for the control tank and 0.85 for the test tank. First column represents the *Aeromonas* species, second column the sampling week and the third column the isolate's code.




XI
XII
XIII
XIV
XV
XVI
XVII
XVIII
A. hydrophila 3 3A11T.1
A. hydrophila 3 3A11T.3
A. hydrophila 3 3A11T.2
A. hydrophila 3 3A11T.4
Aeromonas spp. 4 4A11T.1
A. hydrophila 3 3A8T.1
A. hydrophila 3 3A9T.3
A. hydrophila 3 3A9T.2
A. hydrophila 3 3A10T.1
A. hydrophila 3 3A10T.2
A. hydrophila 3 3A10T.3
A. hydrophila 3 3A5T.3
A. hydrophila 3 3A3T.2
A. veronii 2 2A8T.3
A. hydrophila 3 3A3T.4
A. hydrophila 5 5A8T.3
A. hydrophila 5 5A8T.2
A. hydrophila 5 5A8T.4
A. hydrophila 5 5A8T.1
A. hydrophila 4 4A1T.1
A. hydrophila 3 3A5T.2
A. hydrophila 3 3A5T.4
A. hydrophila 4 4A10T.1
A. hydrophila 5 5A4T.3
A. hydrophila 5 5A9T.1
A. veronii 5 5A4T.2
A. hydrophila 4 4A6T.4
A. hydrophila 4 4A7T.4
A. hydrophila 4 4A8T.1
A. hydrophila 4 4A10T.4
A. hydrophila 4 4A8T.2
A. hydrophila 4 4A11T.4
A. hydrophila 4 4A10T.2
A. hydrophila 4 4A11T.2
A. veronii 2 2A5T.1
A. veronii 3 3A9T.4
A. veronii 2 2A4T.3
A. veronii 2 2A5T.3
A. veronii 2 2A3T.3
A. veronii 2 2A4T.4
A. veronii 2 2A7T.1
A. veronii 2 2A7T.3
A. veronii 2 2A6T.1
A. hydrophila 4 4A11T.3
A. hydrophila 4 4A4T.3
A. hydrophila 4 4A5T.2
A. hydrophila 4 4A4T.1
A. hydrophila 4 4A1T.3
A. hydrophila 4 4A4T.2
A. hydrophila 2 2A1T.1
A. hydrophila 2 2A5T.2
A. hydrophila 2 2A4T.2
A. hydrophila 2 2A3T.1
A. hydrophila 4 4A1T.2
A. veronii 2 2A10T.4
A. veronii 2 2A11T.1


XIX
XX
XXI
XXII
XXIII
XXIV
XXV
A. hydrophila 4 4A5T.3
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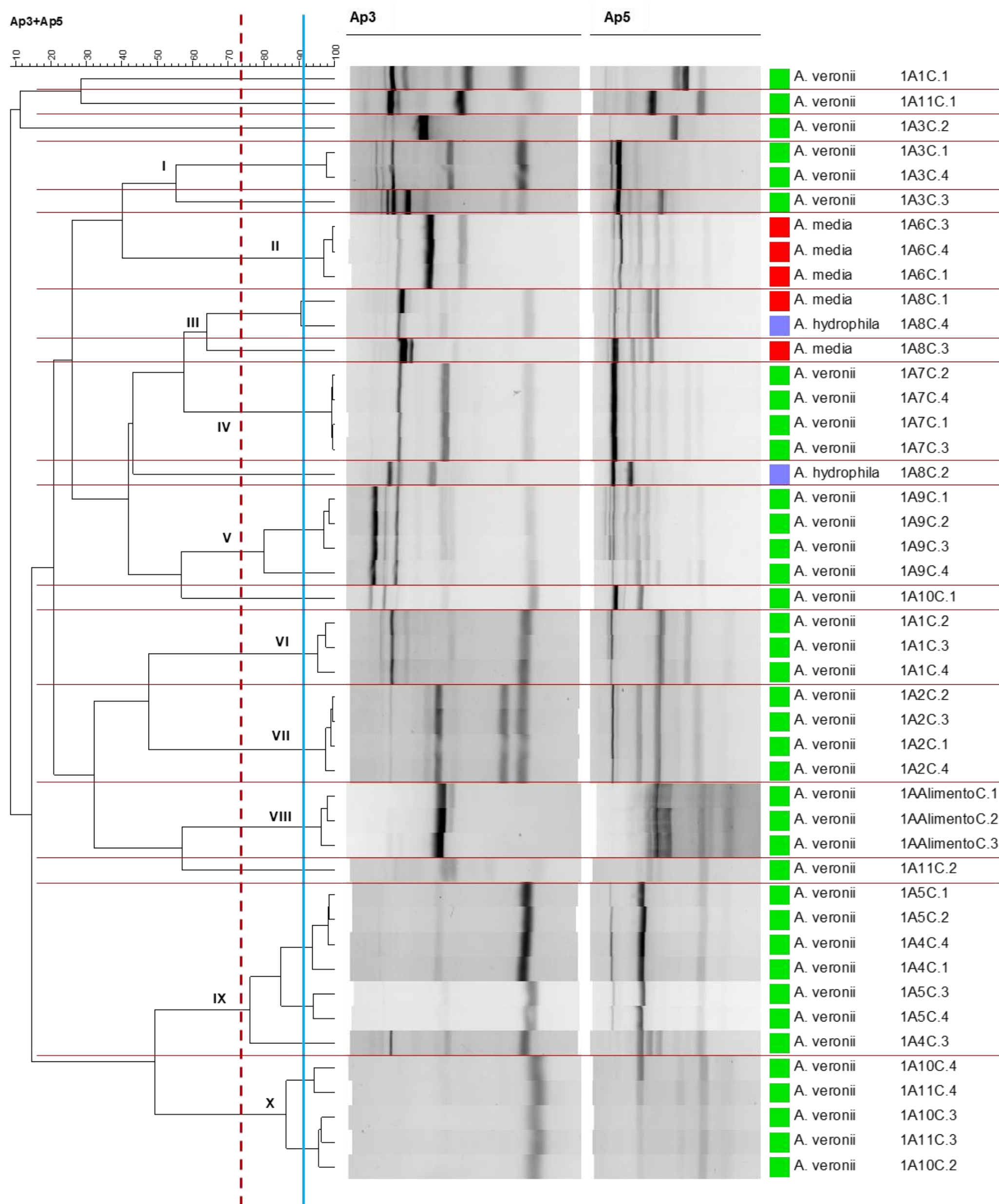
A. hydrophila 4 4A5T.1
A. hydrophila 4 4A7T.3
A. veronii 2 2A7T.2
A. veronii 2 2A9T.1
A. veronii 2 2A8T.4
A. veronii 2 2A9T.2
A. veronii 2 2A3T.4
A. hydrophila 4 4A2T.3
A. hydrophila 4 4A2T.4
A. hydrophila 4 4A2T.2
A. hydrophila 4 4A2T.1
A. hydrophila 4 4A1T.4
A. hydrophila 4 4A6T.1
A. hydrophila 4 4A6T.2
A. hydrophila 4 4A6T.3
A. media 2 2A3T.2
A. hydrophila 2 2A1T.3
A. hydrophila 2 2A2T.4
A. hydrophila 2 2A2T.1
A. hydrophila 2 2A2T.2
A. hydrophila 2 2A1T.2
A. veronii 2 2A6T.2
A. hydrophila 4 4A9T.2
A. hydrophila 4 4A10T.3
A. hydrophila 4 4A8T.4
A. hydrophila 4 4A9T.3
A. hydrophila 4 4A8T.3
A. hydrophila 4 4A9T.1
A. hydrophila 5 5A10T.3
A. hydrophila 5 5A10T.4
A. veronii 5 5A4T.4
A. veronii 5 5A6T.2
A. veronii 5 5A6T.1
A. veronii 5 5A6T.3
A. veronii 5 5A6T.4
A. hydrophila 2 2A10T.1
A. veronii 2 2A11T.3
A. hydrophila 3 3A10T.4
A. veronii 2 2A6T.4
A. hydrophila 2 2A9T.4
A. hydrophila 3 3A3T.1
A. hydrophila 4 4A9T.4
A. hydrophila 5 5A9T.3
A. hydrophila 5 5A9T.4
A. hydrophila 5 5A9T.2
A. hydrophila 5 5A10T.1
A. hydrophila 5 5A10T.2

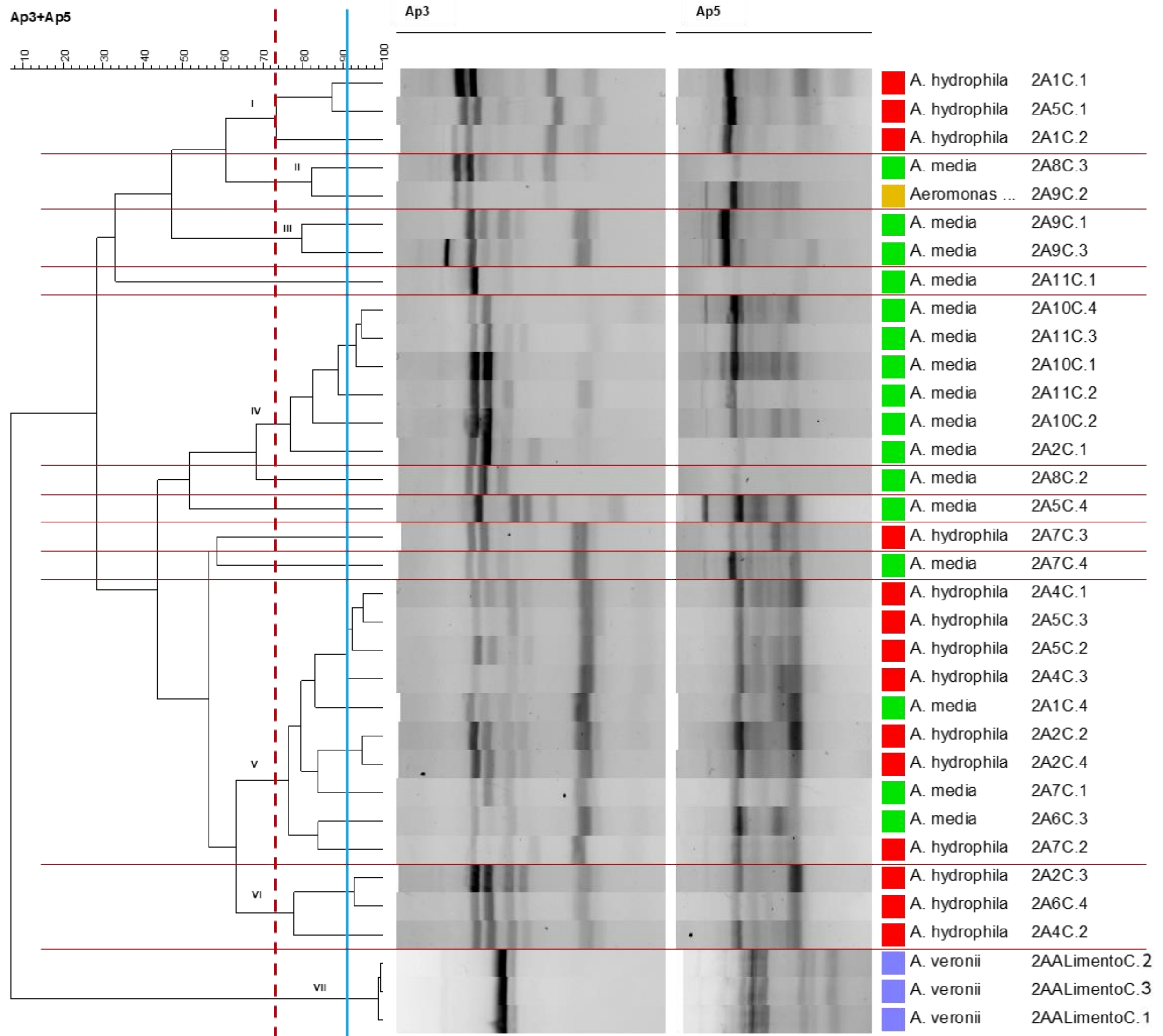
Annex XX

Supplementary Figure S19. Dendrograms of the bacterial collection from the control and test tank in each sampling week based on the composite analysis of RAPD fingerprints with primers Ap3 and Ap5 (Pearson correlation coefficient and UPGMA clustering). Blue line represents reproducibility level (91.88 %). Isolates displaying higher similarity levels were considered identical. Red dash line represents cut off level (62.93 %). Red lines represent cluster division. Cophenetic correlation coefficient was: 1) control tank: 1st week – 0.86, 2nd week – 0.87, 3rd week – 0.93, 4th week – 0.93, 5th week – 0.89; 2) test tank: 1st week – 0.79, 2nd week – 0.87, 3rd week – 0.87, 4th week – 0.89, 5th week – 0.90. First column represents the *Aeromonas* species and second column the isolate's code. Alimento – Food, Cuidador – Aquarist.

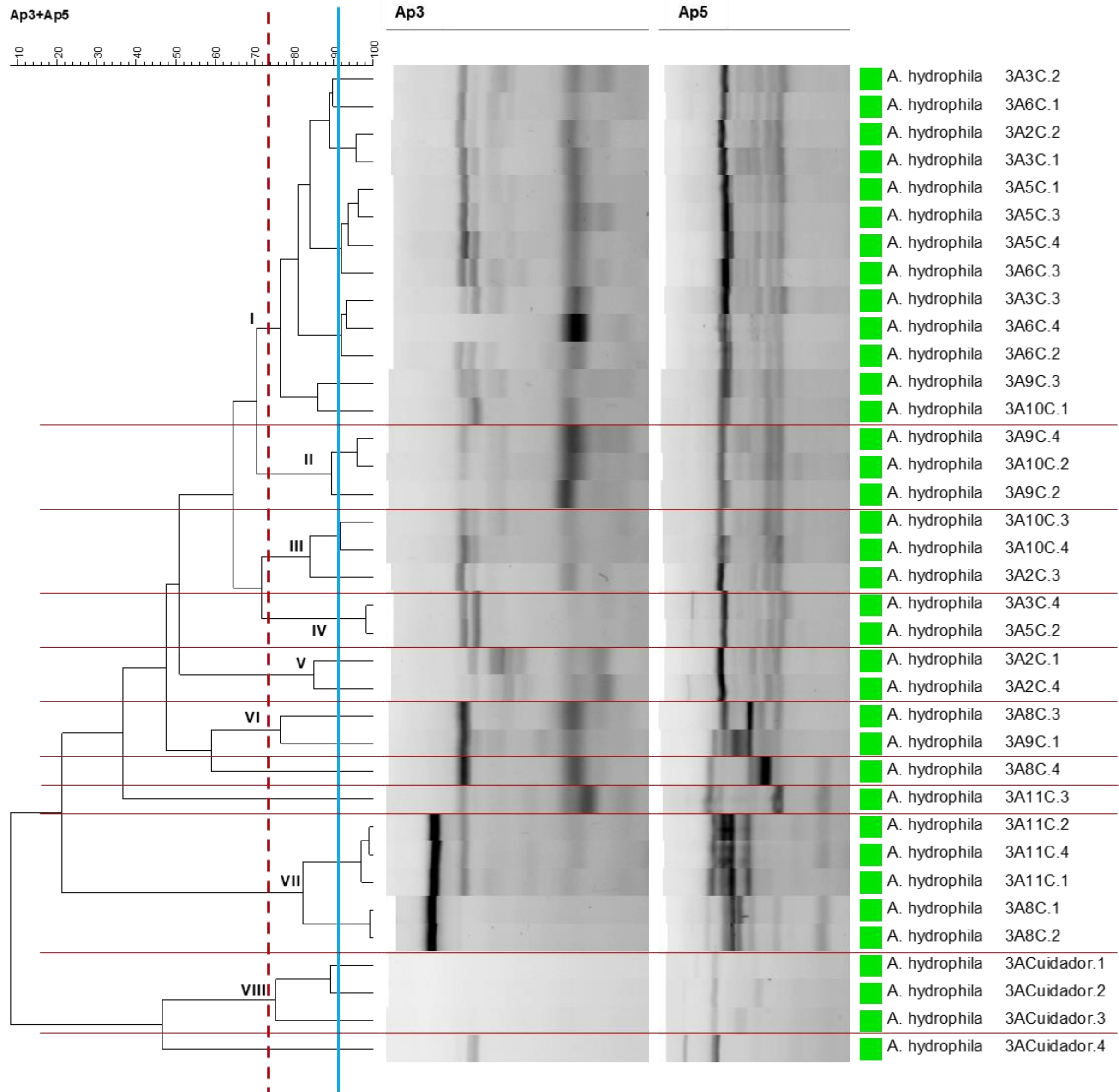
Control Tank 1st Week



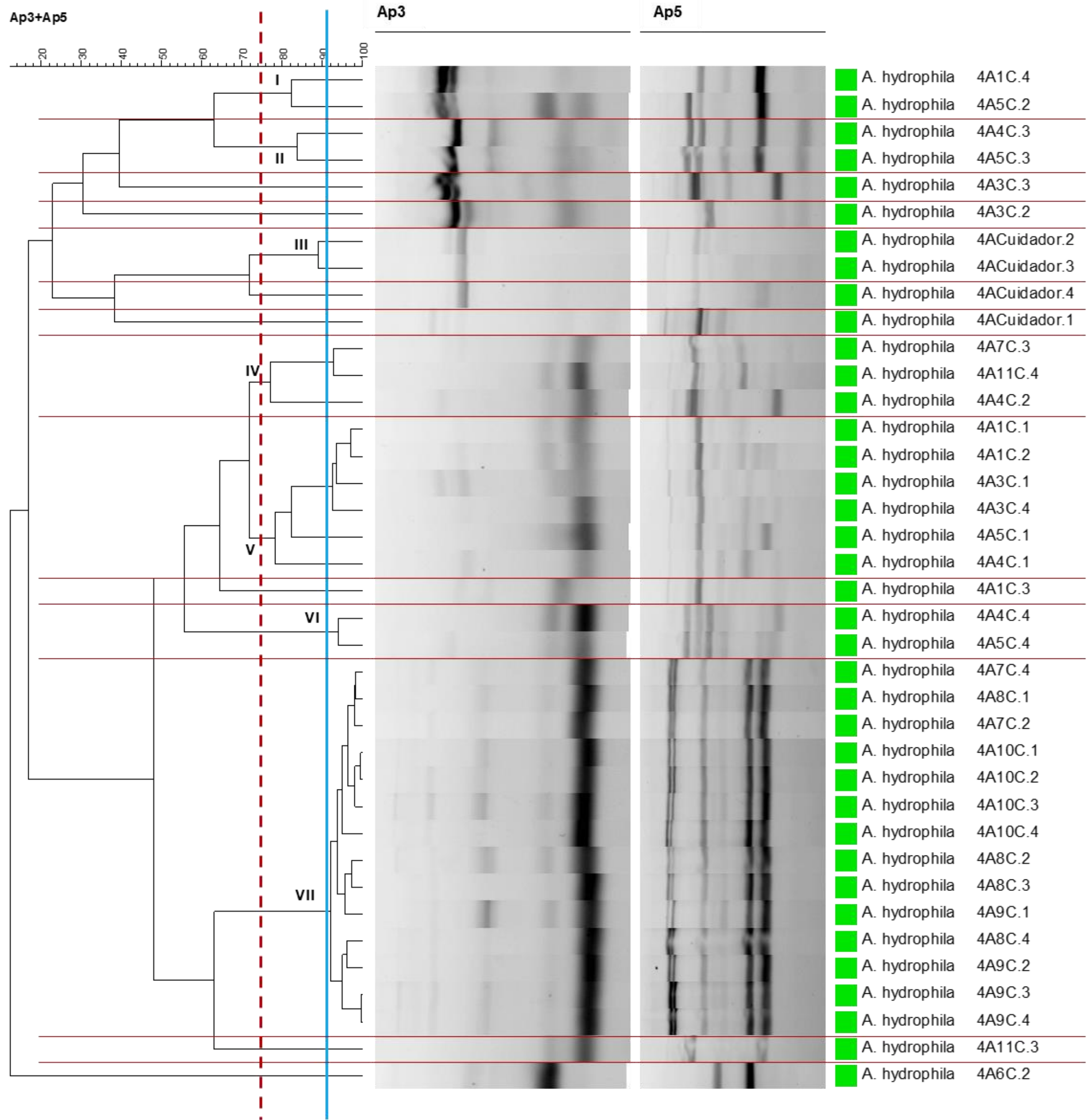
Control Tank 2nd Week



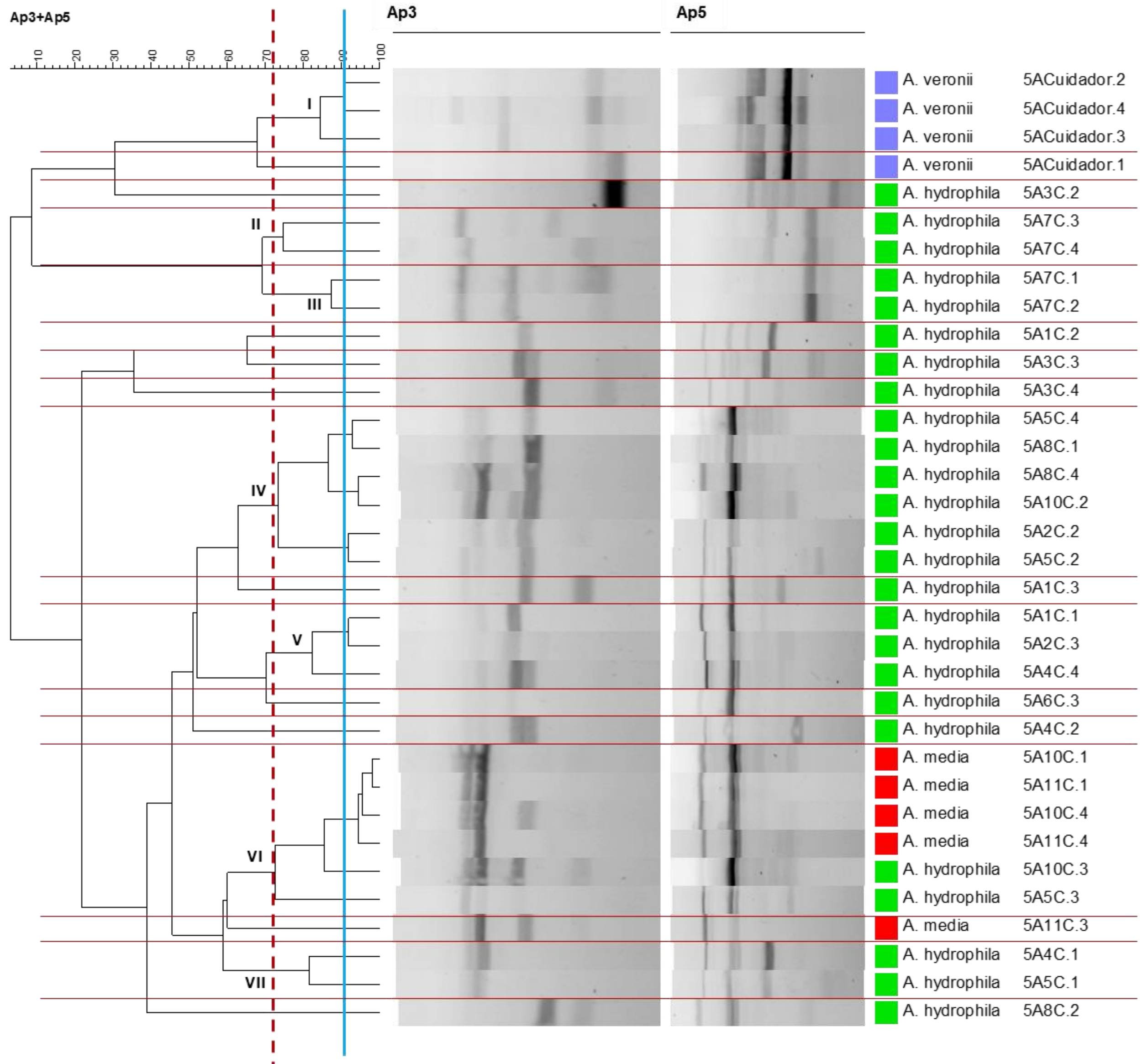
Control Tank 3rd Week



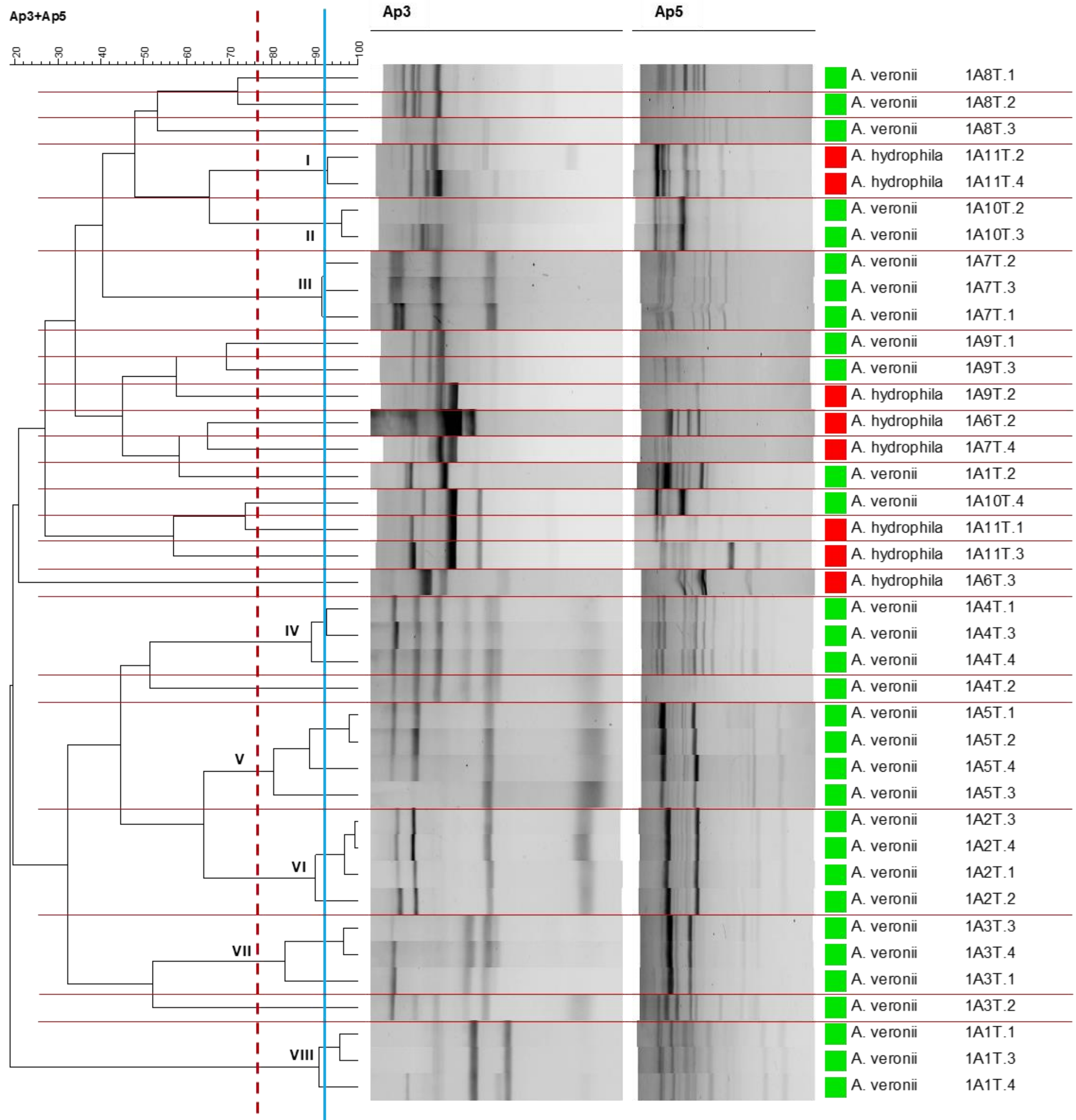
Control Tank 4th Week



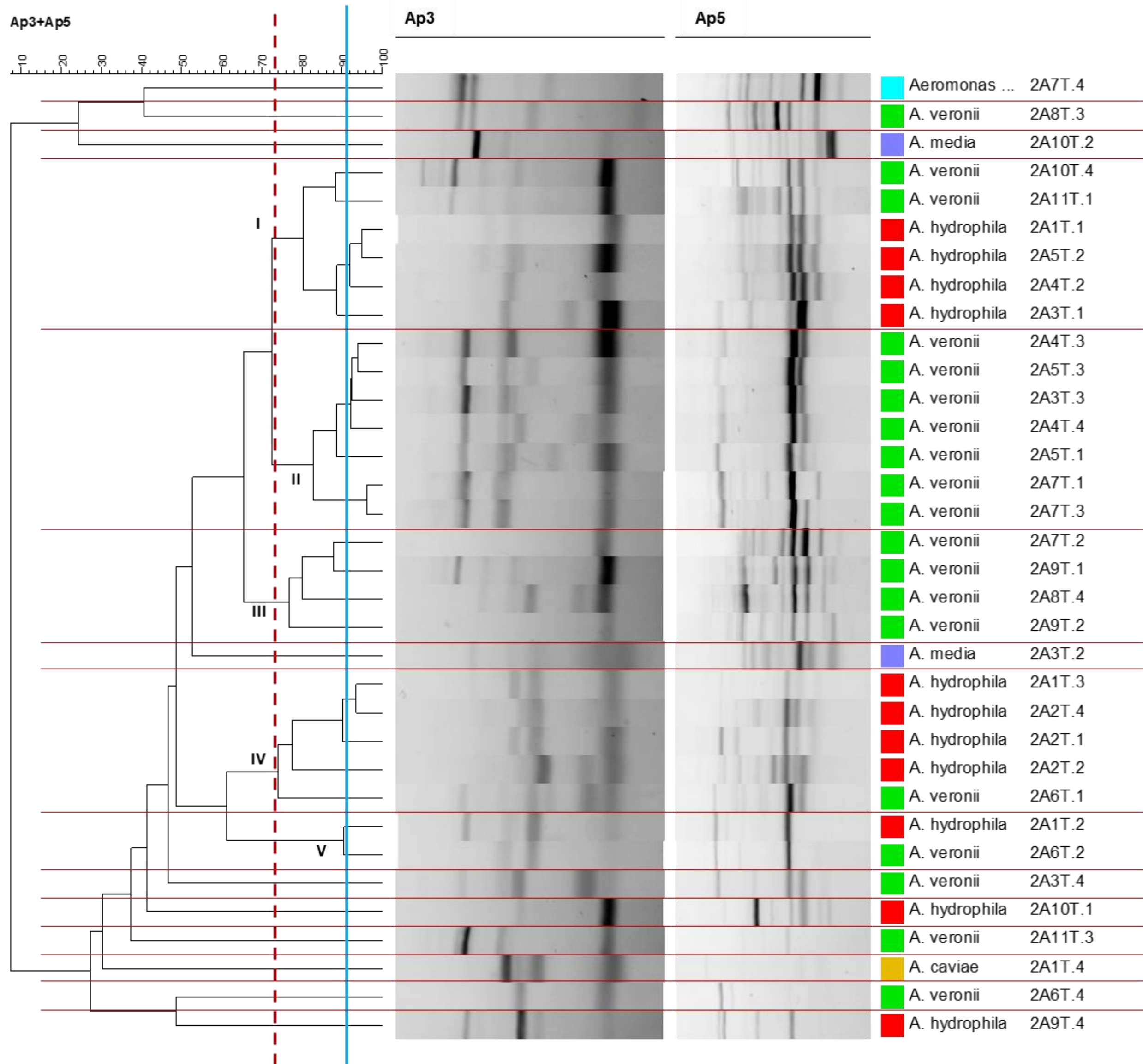
Control Tank 5th Week



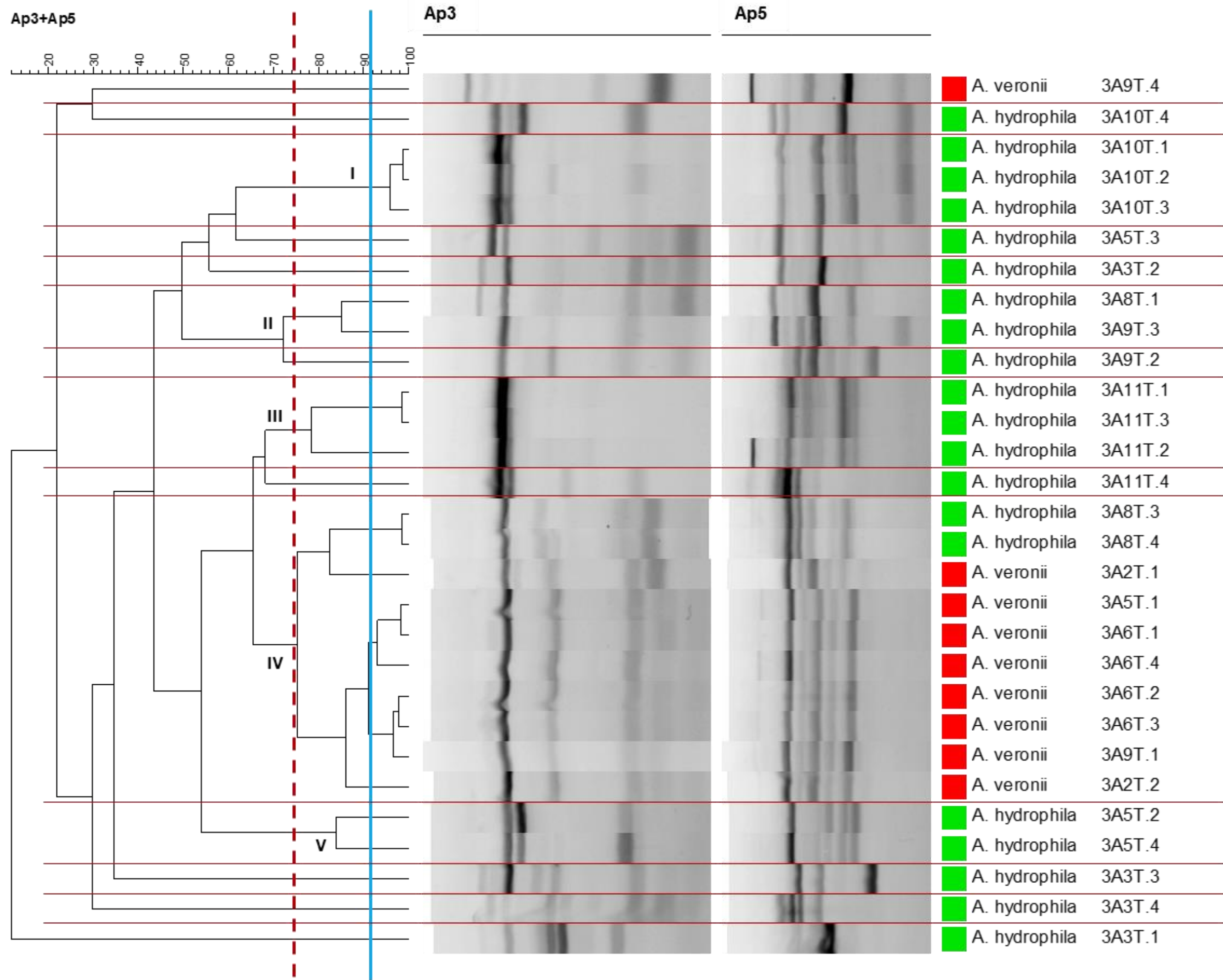
Test Tank 1st Week



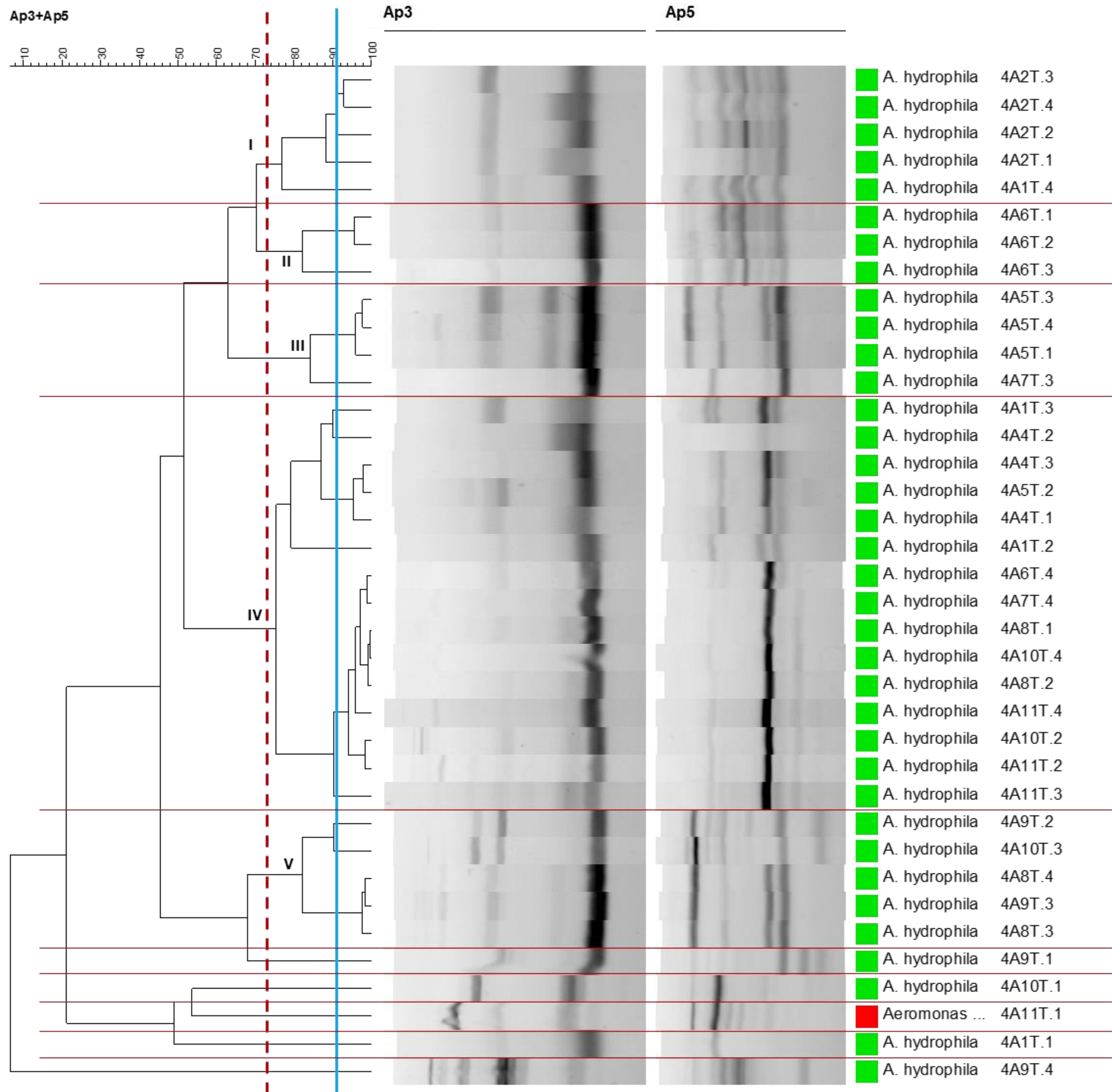
Test Tank 2nd Week



Test Tank 3rd Week



Test Tank 4th Week



Test Tank 5th Week