Universidade de Aveiro Departamento de Biologia
Ano 2017

Fausto Joaquim Ribeiro Sá

Microbiota do peixe, a influência de variações sazonais e contaminação em espécies bacterianas cultiváveis

Fish microbiota, the influence of seasonal variation and contamination on cultivable bacterial species

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Ecologia Aplicada, realizada sob a orientação científica do Doutor Sérgio Miguel Reis Luís Marques, investigador Pós-Doc, do Departamento de Biologia da Universidade de Aveiro e coorientação do Doutor Fernando José Mendes Gonçalves, professor associado c/ agregação do Departamento de Biologia da Universidade de Aveiro

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agradecimentos

Gostaria de agradecer ao meu orientador, Dr. Sérgio Marques, pelo que me ensinou ao longo desta etapa. E também um agradecimento ao professor Dr. Fernando Gonçalves pela sua co-orientação e pela oportunidade de me deixar integrar nesta equipa.
Queria deixar um agradecimento especial à Raquel, à Inês à Joana e ao Carlos, por toda a ajuda que me deram no laboratório e por trazer algum riso à câmara de fluxo e à centrifuga.
Como não podia deixar de ser, muito obrigado aos meus amigos que tanto me aturaram e sempre apoiaram, Fábio, Tiago, Sofia, João, Joana e Cátia. Estiveram no início e estarão até ao fim.
Ao CiRAC e a todos os seus elementos, graças a eles pude distrair-me e manter-me focado e divertido.
À rapariga que mais me ajudou nos momentos bons e maus desta aventura, à minha namorada, Filipa, muito obrigado por tudo.
Aos meus pais e irmã por toda a confiança, apoio e amor sem os quais não conseguiria ter chegado até aqui.
Obrigado a todos.

palavras-chave Microbiota, microbioma, Lepomis gibbosus, pele, diversidade

resumo

O estudo da microbiota presente nos organismos revelou que esta tem um papel extremamente relevante para algumas funções biológicas fundamentais, como a imunidade e a nutrição. Essa evidência originou, nos últimos anos, um aumento no número de estudos focando a interação entre a microbiota e os organismos hospedeiros de modo a esclarecer os mecanismos subjacentes a essas interações. Apesar do crescente interesse e conhecimento nessa área, muito, tal como o papel do meio ambiente na composição da microbiota, ainda é desconhecido. Este facto pode ser de extrema importância quando se considera a microbiota da pele, uma vez que pode está facilmente exposta a alterações ambientais. Isto é bastante relevante quando consideramos animais aquáticos, nomeadamente peixes, uma vez que muitas variáveis afetam atualmente os ambientes dulçaquícolas. Algumas das alterações mais relevantes estão relacionadas com as mudanças climáticas e com a contaminação. De fato, a contaminação é uma das principais preocupações relacionadas aos habitats de água doce, com atividades agrícolas assumindo um papel importante devido à ampla gama de produtos químicos utilizados. Para avaliar os efeitos da contaminação em organismos aquáticos é habitual estudar os efeitos diretos, como o comportamento ou a mortalidade, no entanto, os organismos também podem ser afetados indiretamente. Os efeitos indiretos podem ser, por exemplo, alterações na disponibilidade de alimentos ou mesmo na microbiota da pele, o que pode ter importantes implicações na saúde. De forma a aumentar a nossa compreensão sobre os efeitos da proximidade das atividades agrícolas e também da sazonalidade na microbiota da pele de peixe, avaliámos a diversidade da microbiota cultivável em Lepomis gibbosus na lagoa da Vela (centro de Portugal) em locais com e sem influência da agricultura e em diferentes estações do ano. Após isolamento e identificação das bactérias, através da sequenciação do rDNA 16S os resultados revelaram uma clara diferença entre a diversidade das espécies de microbiota cultiváveis de ambas as estações do ano e entre os locais com diferentes influências da pratica agrícola. Em geral, uma maior diversidade de géneros foi encontrada no Outono, enquanto o local mais distante em relação à atividade agrícola revelou a menor diversidade de géneros quando comparado com o local mais próximo da atividade. Considerando a importância da microbiota da pele na imunidade dos peixes, os nossos resultados sugerem que a proximidade a fontes de contaminação pode influenciar a imunidade dos peixes. Os resultados também sugerem que a sazonalidade também pode influenciar a imunidade dos peixes.

keywords Microbiota, microbioma, Lepomis gibbosus, skin, diversity

abstract

The study of microbiota present in organisms revealed that it has as extremely relevant role for some key biological functions such as immunity and nutrition. Such evidence originated, in the most recent years, an increase in the number of studies focusing the interaction between microbiota and the host organisms to further enlighten the underlying mechanisms of these interactions. Despite the growing interest and knowledge in this area, much is still unknown, such as the role of the environment in the microbiota composition. This can be of extreme importance when considering skin microbiota, which can be easily exposed to environmental alterations. This is quite relevant when considering freshwater dwelling animals, namely fishes, since many variables are presently affecting freshwater habitats. Some of the most relevant alterations are related with climatic changes and with contamination. Indeed, contamination is among the major concerns related with freshwater habitats, with agricultural activities assuming a major role due to the wide range of chemicals used. To assess the effects of contamination in aquatic organisms its usual to study direct effects, such as behaviour or mortality, nonetheless organisms might also be affected indirectly. Indirect effects can be for example alterations in food items' availability or even in skin microbiota, which can have important implications in health. To increase our understanding over the effects of the proximity of agricultural activities and also of seasonality in fish skin microbiota we assessed the diversity of the cultivable microbiota in Lepomis gibbosus in lake Vela (centre Portugal) in sites with and without influenced of agriculture and in different seasons. After isolation of bacteria and identification through 16S rDNA sequencing the results revealed a clear difference between the cultivable microbiota species of both seasons and between the sites with different influence of agriculture. Overall a higher diversity of genera was found in Autumn while the farthest site in relation to the agricultural activity revealed the lowest diversity of genera when comparing with the site closer to the activity. Considering the importance of skin microbiota to fish immunity, our results suggest that the proximity to sources of contamination may influence the immunity of fish. The results also suggest that seasonality may also influence the immunity of fish.

Table of Contents

Chapter I

Chapter II

Chapter III

Chapter I

General Introductions

1. General Introduction

1.1 Introduction

Over the last few years there has been an increasing interest in microbiota from animals and to try to understand their function. Initially the focus was on human microbiota, to try and understand the microbiota function and establish healthy baselines, to differentiate health conditions bacteria for our body and pathogenic bacteria that harms us; this first studies also had the objective to understand the full range of the human microbiota (1). The first works were focused merely on the gut microbiota, but soon they understand that others parts of the human body were also of interest, like the skin, the oral cavity, respiratory tract, and others (1–4).

After sometime this studies started to focus on animals like mammals, birds and even insects, but in the same way this studies started by only paying attention to the gastrointestinal tract (gut) microbiota (5–7). The amphibian class also called the attention of the investigators that wanted to understand how the skin and mucus provided defences to the organism. In many works about this area we can see that the microbiota of the amphibian is determined and changed by early life events, like the own host genotype, genetic inheritance from the progenitors and even by the surrounding environment (8). The work of McKenzie in 2011 determined that amphibian species that lived in different habitats have more alike microbiota than different species that inhabit in the same place, showing us that it exist a transmission from parents to descendants (9).

Soon they turn their attention to fishes, thinking and presuming that their microbiota would reflect that of the water and from the food they consumed (10,11). In this first studies they affirm that the skin microbiota represents the bacteria of the surrounding water because they discovered different bacteria species in marine, estuarine, and freshwater fish species (11); they also turn their attention to the gill microbiota, but assumed that this microorganisms were trapped in the gill filaments as water passes in the gills (11); on early works they concluded that the gastrointestinal microbiota was dependent of the most recent food that was ingested, because the ratio of microbial biomass in the stomach to that in the intestinal contents was almost the same and in some studies they took in consideration that their digestive tract were still fill with food at the time of the surveys (10).

The studies that were conducted had the limitation of only working with the “culturable” sampled bacteria, not having a clear and complete spectrum of the total microbiota diversity of the organism. It is now understandable to the scientists that only 1% of the bacteria that we can observe at the microscope, can grow in the traditional cultural media and conditions that we find in laboratories (12). Many studies have appeared to explain this phenomenon and many conclusions were made. For instance, some of the bacteria can’t grow in laboratory condition because in nature they were in a dormancy state, like endospores, cysts and conidia, and when presented with the nutrient rich media of the cultural plates they can’t adjust and have their grow halted (13). We can also find some bacteria species that have very precise conditions of pH, incubation temperature or levels of oxygen to properly grow (14). The growth of the bacteria could also be affected by

antibacterial substances that are present on the medium, can die by competing with others species in the same plate, can be neglected and excluded by the investigator because sometimes the time of incubation is long and the scientist didn't gave time enough to grow, and finally, because some bacteria are phenotypically indistinguishable between one another and by optical observation of the cultural plates the scientist choose just one of theme, thinking they are the same (15).

To try and resolve this problems and get a full view on the total microbiota diversity inhabiting the various microbiomes that we can find in an organism, the researchers started to make use of a variety of culture-independent methods in the molecular area, like small-subunit rRNA methods, microautoradiography combined with fluorescence hybridization or stable isotope probing (14,16). Thanks to this new line of work the scientific community settled that, the fishes microbiota is extremely diverse, this diversity plays a key role in the organism line of defences, and that the fish microbiota can be influenced by many factors, like temperature, pH, contaminants and many other (12).

If we want to fully understand the full range of the fish microbiota we must address all the different body parts of it separately, the gut microbiota as different aspects than the skin microbiota, and we need to understand the importance of this bacteria to the organism and in which way they are beneficial to them.

1.1.1 Gastrointestinal Microbiota

The study of gastrointestinal microbiota is one of the main areas of interest for researchers studying fish microbiota. This interest is related to the fact that gastrointestinal microbes play an extremely relevant role to the development of the fish immune system (17). The fish gastrointestinal tract was one of the first points of interest for the researches, like in the humans the intestine has a well-known flora associated to it, with an important role in the organism health and development. The estimated count of bacteria that inhabit this organ ranges between 10^7 to 10^{11} bacteria per gram of intestinal content, still, this values depends on the units that are used and we can find different methods on different works (18–20), so it's difficult to compare different researches, but in this paper it will only be addressing the most common phylum and genera's that can be find across the different researches.

The studies on this field tend to divide the species in autochthonous ones, that attach to the intestinal mucosa and allochthones ones, that don't attach (18). To make this differentiation the autochthonous species samples come from intestine, while the allochthones come from the feces of the fish (12).

Feces samples have more microbe's diversity than the intestine, even so they have many species in common. For instance, in both type of samples we can find the following representative genera to freshwater fishes: *Aeromonas*, *Enterobacter*, *Pseudomonas*, *Micrococcus*, and *Bacillus* (18,21–23). On the other side in marine fishes, we find a greater occurrence of *Pseudoalteromonas* and *Vibrio* (24–26), and in the feces samples the most typical genera observed is *Photobacterium* (26,27), besides this, *Pseudomonas* is also found

in the marine species like in the freshwater species, showing both in the intestine and feces, it's an ubiquitous presence in many works and different fish species (18,21–27).

1.1.2 Skin Microbiota

The fish skin is one of the first barriers to prevent the entering of invasive species, we can consider this line of defence part of the innate immune system of the animal (28). Apart from being a physical barrier to invasion, we might even consider the fish's skin as also being a biological barrier, because we can find a wide variety of bacteria that inhabit their skin. We can consider then, the skin a micro environment, a microbiome, that harbours a diverse bacteria community, that we call microbiota (12). We already know that the environment can change and affect the microbiota of the fish in many ways, but other bacteria in the water can also affect this community, adding some species that benefit the animal or killing other bacteria's that were part of the fish defences (19).

Through many works, a large amount of species has been reported, but some of them are common between these researches, like for example, the most usual *phylum* is Proteobacteria, but they also find species from the *phylum* Bacteroidetes and Firmicutes.

In the freshwater fish species we find bacteria from the genera *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Enterobacter*, *Moraxella*, and *Pseudomonas* (20,29–31), while in the marine fish species, we manly find bacteria from the genera *Vibrio*, *Pseudoalteromonas*, and *Photobacterium*, but we can also find *Pseudomonas*, *Aeromonas* and *Acinetobacter* as well (20,24,32).

1.1.3 Gill Microbiota

The fish gills are very relevant for this kind of studies, because it is one of the major paths of infection to the organism, due to its function of gases exchange, to obtain oxygen to the animal from the water (31). Despite of this significance there aren't many studies concerning this theme, but it's still easy to find what are the most common genera discovered.

Again, let's make the separation into fresh water fishes and marine ones. In both instances the most common phylum is Bacteroidetes, but in fresh water we find bacteria from the genera's *Acinetobacter*, *Aeromonas*, *Enterobacter*, and *Pseudomonas* (12,23,31). On marine fishes it's more usual to encounter bacteria from the genera *Photobacterium*, *Pseudoalteromonas*, and *Vibrio* (33–35).

The species found are very similar to the ones found in the skin, and the same difference are found between freshwater and marine fishes, showing us why the first studies believed that this microbiota reflected the bacteria existing in the water (11).

1.1.4 Internal Organ Microbiota

It's not the focus of most of researches, but some works have addressed other organs to study their microbe community. In these organs the bacteria community isn't always associated with healthy fish, but despite that it also has some function of defence to the organism. Many organs have been studied like, the kidneys, blood, ovary, liver, spleen, brain and even light emitting organs in deep sea fishes (24,35–38). There were also works that studied the eye, but they concluded that an healthy fish doesn't appear to have bacteria associated with their eyes (36).

In the internal organs we find I similar profile of bacteria, where Proteobacteria is the phyllo that can be found more commonly, and the other representative phylum are Actinobacteria and Firmicutes. Most of the samples isolated are from the genera *Pseudomonas*, *Aeromonas*, and *Micrococcus* (24,36–38), and in seawater fishes it was also isolated bacteria from the genera *Vibrio* and *Photobacterium* (24,35,37,38).

1.2 Factors influencing the Microbiota

Right from early works it was clear to scientists that bacteria community of fishes shifted because of the environmental parameters like salinity, temperature, precipitation, dissolved oxygen and pH (39). Like it was predicted, many studies saw that the gut microbiota, more than influenced by environmental parameters, it was also influence by the food that was ingested (12,18,29,39) and recent works show that same fish species in different environments exhibit differences in the bacteria diversity, fishes that live in natural habitats show a higher diversity than the ones that live in semi-natural environmental (18). Also in early studies it was evident that the fish mucus could inhibit the grown and attachment of bacteria's that would otherwise contaminate the organism (21,39).

One case that we need to take in consideration when analysing the factors that influence the microbiota is the life stage of the organism. For instance, the fish eggs are quickly colonized (40–42), in the same way the skin and gills microbiota establish very early in the fish life, but on the other hand the gut microbiota only stabilize prior to the first feeding (18,43–45), reaching a really stable position, according to McIntoshet al., at about 50 days post-hatch (40).

1.3 Importance of fish Microbiota

The microbiota of the organisms is part of the innate immune system of the fish and are part of the host growth and health. Early studies only saw the gut microbiota as an important mechanism of defence, but more recent studies also take in consideration the importance of the skin as a first barrier to battle the potential hazard bacteria that want to infect the organism (31). The gut microbiota is also well known to scientist, having influence in the development of an healthy intestine, homeostasis roles and protection against pathogenic bacteria, it also have an important influence in the good growth of the organism, not only on fishes but also in other animals in general (18).

Like in what is found in the human digestive tract, the fish have bacteria in their tract that can metabolize cellulose and other polysaccharides for them, helping the fish obtaining other vitamins and nutrients, that otherwise would be impossible to obtain (46,47). Still regarding nutrition values, some bacteria do more than metabolize compounds, they produce fatty acids that can be used by the organism to produce energy (47).

Some initial works in the skin microbiota found some interesting conclusions on the mobility of the organism. Some bacteria that inhabit the skin are hydrophobic and thus provide a smooth layer between the fish and the rearing water reducing the friction, giving a direct benefit to the fish, reduced energy loss, and also benefiting the bacteria thanks to the good substratum with quality nutrients to the microbe community (48,49).

1.4 Probiotics

The exploration of aquaculture industry is increasing from year to year, but must face a constant battle against diseases that affect their life stock. These diseases decrease the production and affects all the industry so, many solutions have been proposed to fight back, but the more current one is the use of antibiotics (50). This brings worries to the community, having antibiotics present in our food is not view as a good thing, and the increasing cases of antibiotics resistance as also caught the attention of the scientists (51,52), making them search for others solutions, one of them being the use of probiotics.

Probiotic, in a simpler definition, refers to a bacteria that are introduced in an organism with positive effects to their health (51). This bacteria act in various ways, it decrease the pathogens that infect the organism and can even help and produce metabolites that are beneficial to the health and growth of the fish (52).

The criteria's that make the researches chose some bacteria to test as a probiotic, passes through the ability of that bacteria inhibit other pathogenic bacteria. This is important because the chosen bacteria most have that beneficial effect, by producing antimicrobial substances to destroy the infection, or reject it by competing with them for space and nutrition (51–53). To see if the probiotic is really effective in helping the health of the organism, other parameters are observed related to the immune function of the fish; for instance they observe the respiratory burst activity, the abundance and activity of phagocytic cells and the abundance and activity of the enzyme lysozyme (12,54).

In spite of the benefits that the probiotics can give to the organisms, there aren't many studies regarding this subject in fishes, and the scientific community understands that other factors can influence the success or unsuccess of this method, like the water temperature, dissolved oxygen, pH and others, but they all acknowledge that this is of great importance to the animals and to the future of the aquaculture industry, so, more studies need to be done in this field (51,52).

1.5 Goals and scope of the dissertation

Through the years it has been clear that the microbiota have a key role in the health of organisms, particularly on fishes, and it is the combination of this great diversity that make it a great mechanism of defence to the fish and promoter of growth and health (46). The gut microbiota as turned itself a lookout point of investigations, and it is clear that this community is essential to the survival of the organism, its essential to its health, to its growth, to its defences against pathogenic invaders and even to its nutrition (26,44,46).

In parallel, studies about other microbiomes are being done. Studies in the skin fish show us the importance of this first barrier against invasion of alien species to the organism (30,31), and more curiously, show us the importance of this community has an energy cost redactor, improving the flow of the animal through the water (48,49). The gill is an important place to look at, because, a unhealthy microbiota wouldn't prevent the entrance of invasive bacteria through the water to the organism lungs (31,33).

This bacteria community can be easily influenced by a roll of factors like temperature, pH and oxygen available, so it's expected that alterations in the environmental conditions affect the microbiota of the fish, and can bring negative disturbs to its life (18,19). It is of prior importance study the influence in the microbiota provoked by the climate alterations and even study the influence of pollutants in the water, to understand to what point this is affecting the health of the fish.

The fact that the environment changes are affecting the defences of these animals is of great concern, so, the knowledge about the fish microbiota is very important to fully understand how the fish's defenses function and what can affect them, and the bacteria found in their body could have some antibacterial properties and could be used to produce probiotics. The microbiota associated to fishes are vastly underexplored and most of the studies are focused on the gut microbiota leaving the skin microbiota overlooked.

Therefore, the main objectives of this dissertation are:

- Compile the available information about the microbiota of fishes, their diversity and how they are influenced by abiotic parameters;
- Study the diversity of cultivable bacteria in the skin of pumpkinseed sunfish (*Lepomis gibbosus)* and the effects of seasonality and contamination in its composition;
- Establish groundwork for future studies regarding fish innate immunity.

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Chapter II

Seasonal variation of Lepomis gibbosus skin cultivable microbiota, in lake under anthropogenic stress

2. Seasonal variation of *Lepomis gibbosus* skin cultivable microbiota, in lake under anthropogenic stress

Abstract

Environmental contamination is, presently, one of our main concerns. There are various sources of contamination but one of the most concerning is agriculture, due to a wide range of chemicals used, that frequently end up in water masses affecting a wide range of aquatic organisms. Usually the effects studied on the organisms are direct effects, such as behaviour or mortality, nonetheless organisms might also be affected indirectly. Indirect effects can be for example alterations in food items' availability or even in skin microbiota, which can have important implications in health.

Fish are one of the groups of organisms that are greatly exposed to contamination with most of the studies focusing direct effects of contaminants and overlooking indirect effects of contaminants in this group. Considering that microbiota can be of extreme importance for a good immunological status it is important to comprehend how contamination can affect it. In this work, we assessed the effect of seasonality and proximity to sources of agricultural contamination in the diversity of cultivable bacteria of the skin of the pumpkinseed sunfish (*Lepomis gibbosus*) from Lake Vela, Portugal.

With this work we found a high diversity of bacteria, from different phyllo (*Actinobacteria*, *Proteobacteria*, *Firmicutes* and *Bacteroidetes*) and genera. The differences between two different seasons of the year are noticeable, and even between two different areas with different water conditions these differences are visible.

2.1 Introduction

Presently we are facing an overall decline of freshwater quality resulting from various sources of contamination such as industrial or agricultural activities. The latter, due to its generalized activity and is one of the main sources of diffuse contamination. As an aquatic life form, fishes are in constant threat and dependence of what surrounds them, and not only the contaminants have a great impact in their health, but also the microorganisms that cohabitate with them can interfere in the wellbeing of these animals.

Microbes can interact with fishes, infecting them if they find the right conditions to do so, and even though most of these bacteria are harmless to them, some are pathogenic and can lead to diseases and ultimately fish's dead. Normally, the animal can resist the infection and survive through multiple mechanisms of the immune system. These responses can be either from, the innate defence system or from the acquired or specific defence system (1). The acquired immune system are humoral responses provided by the production on antibodies or by the cellular immune responses that destroy the foreign agent. On the other hand, the innate or natural immune system is formed by a series of humoral and cellular elements, like for

example the eyelashes of our eyes, the gut microbiota and the skin that prevent the invading species to enter the organism.

The innate system has a very important role for fish species, preventing the invasion and proliferation of microbes, and this efficiency occurs because of three characteristics. One of them being the fact that this kind of defence doesn't depend upon recognition of molecular structures of the microbe, like what happens on the production of antibodies. Also, it's a much quicker response than the production of immunoglobulins. In addition, this type of immunity doesn't depend on the temperature (2). Thus, the innate immune system is very important to fishes in comparison with the acquired one, because the production of antibodies takes a longer time and depends so much on the temperature, they have to be able to defend themselves quicker and with more efficiency (1,2). One of this important barriers of defence for the fish, and the first line of defence is the scales and epidermis, which prevents the infection and spread of bacteria into the fish (3).

Apart from being a physical barrier to invasion, we might even consider the fish's skin as also being a biological barrier, because we can find a great bacterial diversity that inhabit their skin. We can consider then, the skin a micro environment, a microbiome, that harbours a diverse bacteria community, called microbiota (4). Early studies only focused on the microbiota of the gut, and neglected the microbiota of other tissues, like the gills, skin and mucus. Also the first studies on skin microbiota asserted that the bacteria found in the skin reflected the community present in the water (5,6), but recent studies affirm that the fish microbiota has a high diversity, plays a relevant part in their health and can be affected by various environmental factors (4). Most of the bacteria that these studies identify belongs to the *phylum* Proteobacteria, but they also find species from the *phylum* Bacteroidetes and Firmicutes. In the freshwater species we find bacteria from the genera *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Enterobacter*, *Moraxella*, and *Pseudomonas* (7–9), while in the marine species we manly find bacteria from the genera *Vibrio*, *Pseudoalteromonas*, and *Photobacterium*, but we can also find *Pseudomonas*, *Aeromonas* and *Acinetobacter* (10–12). This microbiota as a very important role in defending the fishes against invasive species that could be pathogenic, by competing with them and having antibacterial properties towards some species, preventing them from attaching the fish. Some studies even used this microbiota as a biological tag to know the origin of the fish (13), and to track down where the fish was packaged for market (14).

It is also known that the environment surrounding the organism influences the microbiota and some of the most relevant environmental parameters influencing it are: the levels of dissolved oxygen, temperature, salinity and contaminants in water. Even other bacteria present in the water can change and influence the microbiota, adding some species that can be beneficial to the fish, or killing species that were part of the fish's defences (15).

The knowledge about the fish microbiota is very important to fully understand how the fish's defences function and what can affect them, and the bacteria found in their body could have some antibacterial properties and could be used to produce probiotics. The microbiota associated to fishes are vastly underexplored and most of the studies are focused on the gut microbiota leaving the skin microbiota overlooked. Also, to our knowledge, there are no other studies in Portugal concerning the fishes' skin

microbiota. Therefore, this study will focus on the characterization of the cultivated microbiota of the skin of an invasive fish species (*Lepomis gibbosus*) in lake Vela. We will also compare two different places inside that lake, that have different concentrations of contaminants, and two different seasons of the year (Spring and Autumn) to try and understand what differences we will find in the bacteria species discovered, related to different climate and water pollution.

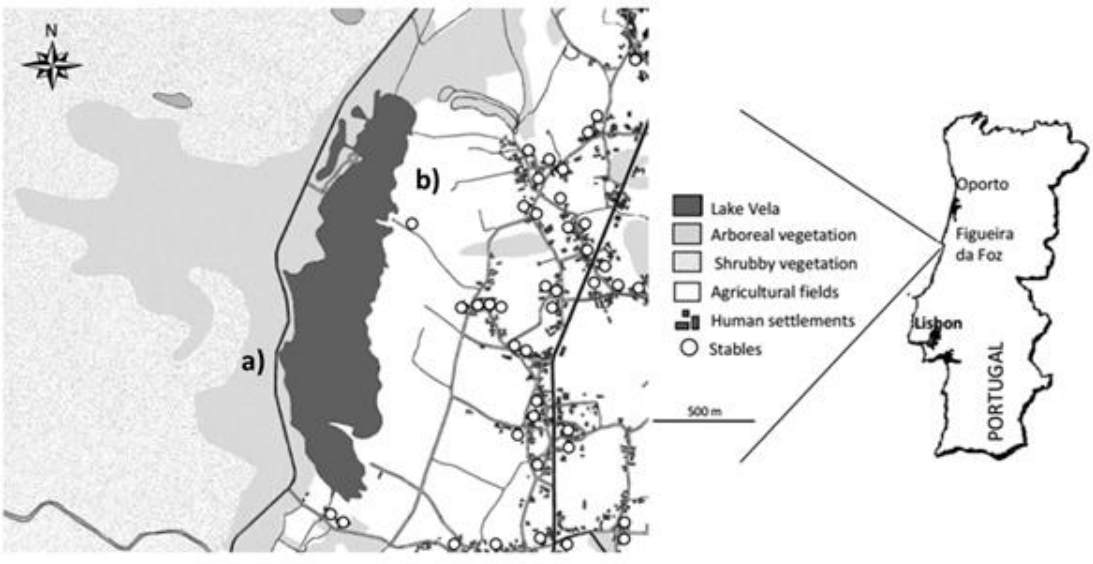
Despite knowing that, for detecting the full spectrum of the diversity living in the fishes' skin we can't rely on culture based methods, for this study, we choose to use them, because this study intends to serve as groundwork for future studies where the objective is to: to i) assess the antimicrobial activity of the bacteria against pathogens, namely against fungus of the genus *Saprolegnia*, pointed as being responsible for a recent fish mass mortality event in Lake Vela; ii) understand how bacteria inhabiting the fish skin, protect it against pathogenic bacteria; iii) understand how pollutants spread across the ecosystem influence the interaction of these bacteria with their hosts and pathogens. Considering these future objectives, we need to have knowledge of the this microbiome (16,17).

2.2 Material and Methods

2.2.1 Study site

Lake vela is a shallow lake, located in the littoral centre of Portugal with an approximate surface area of 70 ha being part of a system called "Quiaios Lakes" (18). Due to of its value and interest as a natural habitat, Lake Vela was included in the Ecological European Net- Rede Natura 2000 (18). Nonetheless, when considering fish community, this lake presents a low diversity, being composed mainly by exotic species such as Lepomis gibbosus, Cyprinus carpio and Gambusia affinis, being Lepomis gibbosus the most abundant (19,20).

From an input of contaminants perspective, the lake can be divided in two distinct areas (fig.1) being mostly related to the agricultural usages of the margins: an agricultural zone and a pine tree forest zone. Studies performed in the lake (18,21)indicate that the agricultural margin is more contaminated than the arboreal one, mainly owed to the use of pesticides and fertilizers in the agricultural activity (18,21).



2.2.2 Pumpkinseed sunfish (*Lepomis gibbosus*)

The pumpkinseed sunfish was introduced in Europe in the latest 19th century and it became one of the most successful introduced fishes (24). The pumpkinseed sunfish is natural from North America (22,24) and it's responsible for the decrease of other fish species in Europe (24). This specie prefers small, shallow, man-made or natural lakes (24,25) and are well known for their territoriality and for that reason they are considered responsible for the decrease of many fish, frogs, gastropods and others species (25). Studies made in Spain show that the more aggressive individuals are the small pumpkinseed, rather than the medium and large individuals, "reflecting an ontogenetic behavioural shift from low to high aggression intensity" (26). The choice for using *Lepomis gibbosus* in this work is due to its territoriality which leads these organisms to life in the same territory for long periods of time. This trait is essential to perceive how the proximity to a contamination source can affect differently the cultivable microbiota from fishes inhabiting the same lake.

2.2.3 Field collection procedures and microbiota sampling

2.2.3.1 Water sampling and chemical analyses

The sampling strategy for this study consisted in sampling fish microbiota from both margins on two different seasons, Autumn and Spring. For each sampling period the pH (WTW330/SET-2 pH meter), dissolved oxygen (WTW315i/SET Oxi meter) and conductivity (LF 330/SET conductivity meter) were measured in each margin and water was collected in amber glass bottles for quantification of six commonly used pesticides. The pesticides selected, and the analytical method were: terbuthylazine, tebuconazole, dimethomorph and glyphosate through LC/MS/MS and deltamethrin and chlorpyrifos through GC/MS.

2.2.3.2 Fish sampling

Adult fishes, five per site and season, with a total body length higher than 10 cm, were captured in two different seasons (Autumn and Spring) in both west margin (arboreal zone) and east margin (agricultural zone). Adult fishes were chosen for sampling microbiota due to increased probability of having a more stabilized microbiota since they would have already reached sexual maturity indicating an age of, at least, two years, through which they would have experienced several environmental variations. Fish were captured using a combination of electrofishing, seine nets and dip nets, and in each season the sampling was performed in

two consecutive days, one per each margin. Captured fish were placed in a 20L plastic bucket with water from the sampling site and aerated, and then microbiota sampling proceeded.

2.2.3.3 Microbiota sampling

It's important to highlight that the same method of microbiota sampling was used in both areas to maintain the same conditions and don't influence the results. Fish were individually removed from the bucket with the help of a hand net previously washed with 70% alcohol and rinsed with sterile distilled water. Fish were handled with nitrile gloves, similarly treated to the hand nets. Every animal, before microbiota sampling, was washed three times with sterile distilled water to remove transient bacteria. A fourth wash was done for the first three sampled fishes, in each site, and the water collected for further inoculation to assess the effectiveness of the wash in removing transient bacteria. After the wash the fish was placed on its left side to proceed with the swabbing on the right side of the body. The swabs consisted in passing three times the cotton tip of the swab through the fish skin, in the same direction as the scales, from the operculum to the caudal peduncle. Immediately after the swab was used to inoculate plates (27). Three swabs were made per fish and inoculated each on one of the following media: Tryptic Soya Agar (TSA), Plate Count Agar (PCA) and Tryptic soy Broth (TSB). A control swab was also made to test possible aerial contamination. This swab was held in the air for a few seconds and then inoculated in a micro tube with TSB.

2.2.4 Isolation and identification of bacteria

2.2.4.1 Isolation of bacteria

In the same day of the sampling microbiota sampling, immediately after arriving to the laboratory, the microtubes containing the TSB inoculated with the fish swab were briefly placed in the vortex and 100 µL were immediately transferred to a new plate with TSA and inoculated with the help of sterile beads. Then all the plates were incubated for 6 days at a constant temperature of 21°C. Whenever bacterial colonies appeared, they were visually compared according to the colour, size, border and texture and if different bacteria appeared, they would be isolated and inoculated in a new petri plate with the respective medium. After complete isolation (fig 2) the isolates were preserved in 30% glycerol at -20ºC for future work. These steps were all carried out in a laminar flow chamber.

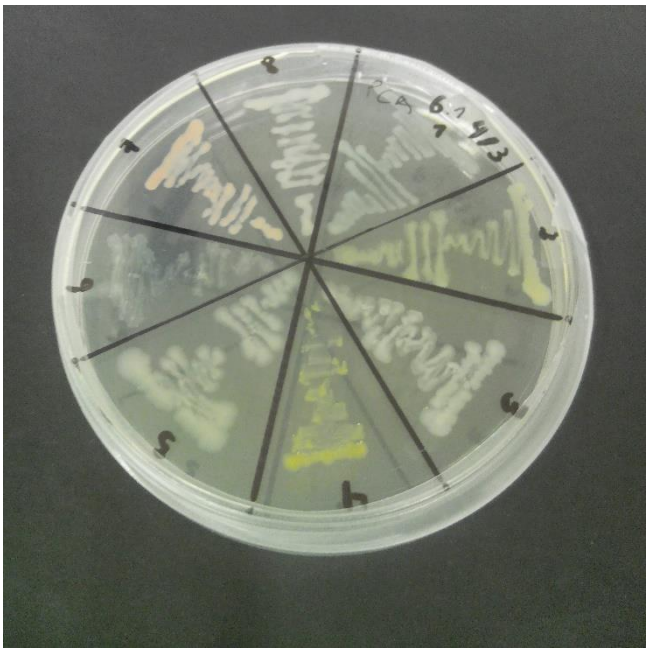


Fig. 2. Plate with isolated bacteria.

2.2.4.2 Identification of bacteria

To identify the bacterial isolates the amplification of the 16S rRNA was performed. First DNA extraction was performed on isolates grown overnight in sterile Eppendorf tubes with 1.0 ml TSB. Briefly, for the DNA extraction, 100 µL of each culture were placed in new sterile tubes and centrifuged for 15 minutes at 15 000 G, discarding the supernatant in the end. Afterwards, after resuspending the pellet in 40 µL of sterile distilled water, a new centrifugation was performed at 15 000 G for 10 minutes. The supernatant was again discarded and resuspend again with another 40 µL of sterile distilled water and incubated for 10 minutes at 100º in a heating block. Afterwards the sample was centrifuged for one minute and preserved on ice. After DNA extraction, we proceeded with the DNA amplification process.

For the 16S rDNA PCR amplification prior to the sequencing the reactions were performed in 25 µL reactions containing 0.2 µM each primer (27F and 1492R) 1x PCR buffer, 0.2 mM each dNTP, 2 mM MgCl2,1U Taq polymerase and 2 µl of cell lysate as template DNA. PCR conditions were as follows: initial denaturation at 94 ºC for 5 minutes, followed by 34 cycles of 94 ºC for 1 minute, 54 ºC for 1 minute, and 72 ºC for 2 minutes, ending with a final extension at 72 ºC for 10 minutes.

Finally, the PCR products were analysed by electrophoresis on agarose gel (1%) stained with SYBR® Safe DNA gel stain to confirm the amplification of the 16S rDNA. The electrophoresis ran for one hour at 90V and 400 mAh.

To identify the bacteria species, the 16S PCR products were sent to the company, STAB VIDA, to be analysed by sanger sequencing. Afterwards, the sequencing data was checked in Chromas Lite software and the sequences were compared with databases, using BLASTn from the National Center for Biotechnology Information (NCBI). introduced in the Standard Nucleotide Blast database to identify them. Later the bacteria species were grouped taxonomical at the genera level.

2.3 Results

The abiotic parameters (table 1 and table 2) obtained within each season didn't reveal a great difference between the arboreal zone and the agricultural zone. Between seasons the most striking difference is in the water temperature, with practically 6 degrees differing between the sampling days. Also, pH values decreased one unit between Spring and Autumn.

Table 1. Water parameters on Spring sampling

Zone	Temperature ºC	pH	O_2 mg/L	Conductivity (µS/cm)
Arboreal	19.5	9.05	12.35	438
Agricultural	22.9	9.07	10.58	413

Table 2. Water parameters on Autumn sampling

Zone	Temperature ºC	pH	O_2 mg/L	Conductivity (µS/cm)
Arboreal	13.2	8.32	10.57	606
Agricultural	13.0	7.94	8.8	581

The results from the chemical analysis of the pesticides (table 3 and table 4) showed that in these two sampling seasons there are pesticides being introduced in the water, namely glyphosate and tertbuthylazine.

Table 3. Pesticide concentration in the Spring for both the arboreal area (Sarb) and agricultural (Sagr)

Pesticide	**Sarb (µg/L)**	**Sagr (µg/L)**
Chlorpyrifos-ethyl	<0.005	<0.005
Terbuthylazine	<0.005	0.006
Deltamethrine	<0.08	<0.08
Tébuconazole	<0.005	<0.005
Dimethomorphe	<0.005	<0.005
Glyphosate	0.044	0.074

Table 4. Pesticide concentration in Autumn for both the arboreal area (Aarb) and agricultural (Aagr)

Pesticides	**Aarb (µg/L)**	**Aagr (µg/L)**
Chlorpyrifos-ethyl	<0.005	<0.005
Terbuthylazine	<0.005	<0.005
Deltamethrine	<0.08	<0.08
Tébuconazole	<0.005	<0.005
Dimethomorphe	<0.005	<0.005
Glyphosate	0.052	<0.02

From the fish sampling, a total of 140 bacteria were obtained in Spring and 147 in Autumn (table 5). Despite not being relevant the differences between the two seasons, in the same season and between the two different areas, some differences were observed. In Spring, each fish had in average of 12 isolates in the agricultural area and 13 in the arboreal site. On the other hand, in Autumn, we obtained an average 19 isolates per fish from the agricultural area and 11 for the arboreal.

Table 5. Number of isolated bacteria by season and area of study

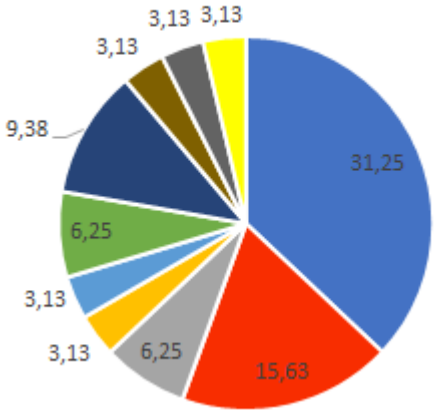
	Area	Nº bacteria isolated
Spring	Agricultural	49
	Arboreal	91
Autumn	Agricultural	94
	Arboreal	53

From the total of 287 bacteria isolated and preserved, only 104 were able to be successfully used in the rest of the work, since only from these ones was possible to extract DNA and amplify it for enabling its' sequencing. This discrepancy in the values was due to the impossibility of extracting the DNA of some bacteria with this method, some of the bacteria also didn't grow after being preserved.

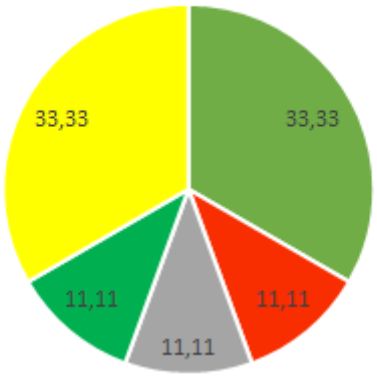
After sequencing 100 bacteria were identified, and other 4 were not successfully sequenced. Overall the bacteria phylum that were identified were *Actinobacteria*, *Proteobacteria*, *Firmicutes* and *Bacteroidetes*. At this level, the only difference observed was between the two seasons, in Spring it was identified bacteria from the phylum *Firmicutes*, while in Autumn it wasn't and *Bacteroidetes* was found. After grouping the bacteria by genera (fig. 3) the results revealed a trend for higher diversity in terms of genera in Autumn for both areas, with the arboreal site presenting six more genera and the agricultural site three more. Also, when

comparing sites within the same season, animals from the agricultural area presented always higher diversity of genera.

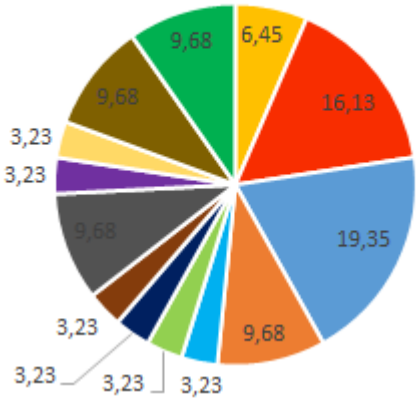
Graphic 1.1. Agricultural area, Spring season



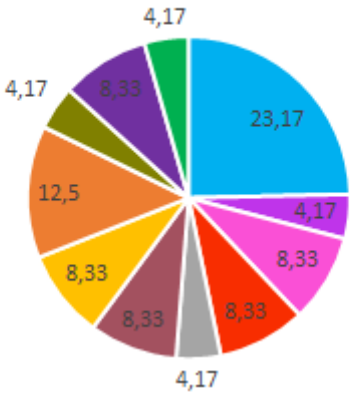
Graphic 1.2. Arboreal are, Spring season



Graphic 1.3. Agricultural area, Auntumn season



Graphic 1.4. Arboreal area, Autumn season



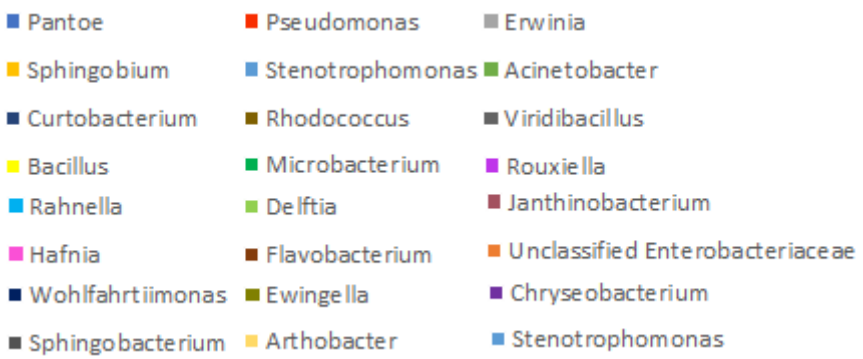


Figure 3. Charts showing the percentage of isolated bacteria, by genera, in the two areas and seasons

2.4 Discussion

In the present work we considered two different areas within lake Vela, according to the land use near the lake's margins. Taking land use into consideration, we would expect different concentrations of pesticides between the arboreal site and the agricultural site. Nonetheless, in Spring the concentration of glyphosate in the arboreal area was almost half of the concentration in the agricultural area, having, nonetheless, concentrations within the same order of magnitude. On the other hand, in the Autumn, glyphosate was only detected in the arboreal site, which was totally unexpected since historically (18,20) when pesticides were detected in this west margin, the east margin would always present higher concentrations. Nonetheless, the increase of this pesticide in the autumn could be a due to the migration with the time and the winds in this season that move the water mass.

Taking in consideration the total number of bacteria isolated in Spring and in Autumn we didn't find relevant differences between these two seasons (140 and 147 bacteria), but considering the two different areas separately, it's possible to perceive some differences. In Spring a higher number of bacteria was isolated in the arboreal side while in the Autumn more bacteria were isolated in the agricultural area. These differences may occur due the many factors that influence the growth and development of the microbiota, like the temperature, percentage of oxygen available and pH, which all changed between different seasons. Also, another possibility is that, the difference in abundance in Spring, can be related with the use of fertilizers and pesticides in the agricultural area, affecting the microbiota of the fish skin (28). Indeed, the quantification of the six selected pesticides, showed that the herbicide glyphosate was present in higher concentrations in the agricultural area in Spring. While some bacterial strains are highly resistant to glyphosate, others are extremely sensitive and, depending on the concentration, can be inhibited by this herbicide. Thus, this could explain the difference in the number of isolates between the agricultural and arboreal areas. Nonetheless, when considering the number of genera identified on both sites, the trend was the opposite, for both seasons. In the case of Spring, this can be related to the fact that most of the isolates, that could not be cultivated after preservation, were from the arboreal site sampling, which lead to the probable loss of some genera. Nonetheless, the higher number of genera in the agricultural site, both in Spring and Autumn, can be also related to the higher levels of nutrients and organic matter found already in lake Vela (18, 20).

This work only revealed 4 phyla inhabiting the skin of the fishes, *Actinobacteria*, *Proteobacteria*, *Firmicutes* and *Bacteroidetes*, these are well reported in others works regarding freshwater fishes (7–9). The most common bacteria genera found are: *Pseudomonas*, *Acinetobacter*, *Rahnella*, *Stenotrophomonas*, *Bacillus*, *Rewinia* and *Curtobacterium*.

Pseudomonas was the most prevalent genus found in the work, it was found in both season in the two sampling sites, the arboreal area and the agricultural area. Bacteria from this genus are very common, being found in all beings and in, water, air and soil. Many species in this genus are associated with diseases (29,30). Nonetheless, none of the species identified in our study, have been reported as pathogenic, namely for fishes.

Overall, our results indicate that there are differences in the cultivable microbiota diversity between sites. This indicates that these two different land usages, near the lake, must surely have and important role in modulating skin microbiota. This can ultimately represent different susceptibilities to pathogens in the same lake, depending on the area where fish inhabit. In the future

further studies should be carried out to try to understand how can other factors, besides the abiotic ones, like the contaminants existing in the water, affect the fish's defences. Also, further studies should be undertaken to understand if the bacteria found in the pumpkinseed fish skin, have antibacterial capacities towards fish pathogens.

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Chapter III
Final Remarks

3. Final Remarks

It's clear that the microbiota has a major role in the health of organisms. In fishes it's perceivable that the bacterial community plays a major role in various physiological processes, including nutrition and growth and in immunity. These roles depend on the respective microbiome (1–4).

These communities can be easily affected by a high range of factors, such as temperature, pH and oxygen availability. Considering this susceptibility to environmental parameters it is expected that global climate changes together with environmental contaminants will have a negative impact in these communities, and thus, affect the immune defences of the animals (5,6). Also, knowing that microbiota is vulnerable to environmental alterations, it is expected that contaminants also play an important role in its structure. Thus, it is of utmost importance to increase the knowledge regarding the influence of climate alterations and contamination in the microbiota, to understand the extent of the effects on fish.

Reinforcing this idea, our study revealed that between the different sampling seasons there were differences in the diversity of the cultivable microbiota from skin. These differences may have occurred due to the environmental factors that influence the growth and development of the microbiota, like temperature and percentage of available oxygen, which changed between seasons. Other possibility is that, the differences resulted from differential use of pesticides and fertilizers between season, and sampling sites, affecting the fish skin bacterial community, as observed by Zhao and co-workers (7).

Considering the possible implications of alterations in the microbiota diversity it is of extreme importance to further study the effect of contamination in the microbiota diversity through controlled laboratorial exposures. Furthermore, the antimicrobial activity of cultivable microbiota, against fish pathogenic agents, should be assessed, as well as, the influence of contaminants in its' efficiency against the pathogenic agents. Ultimately, and in a similar way to the studies carried out with amphibians, such approach could lead to the development of probiotic agents that could be used to reduce mass mortality caused by pathogenic agents (8).

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