Universidade de Trás-Os-Montes e Alto Douro

Lactococcus lactis RBT18 isolated from rainbow trout: antimicrobial activity, taxonomic identification, bacteriocin purification and mass spectrometry analyses

Dissertação de Mestrado Integrado em Medicina Veterinária

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“Educate and inform the whole mass of people, they are the only sure reliance for the preservation of our liberty.”

Thomas Jefferson

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ABSTRACT

As aquaculture is recognized as the fastest-growing food-producing sector worldwide, new obstacles and challenges emerge derived from its intensification. In this respect, infectious diseases, such as lactococcosis caused by *Lactococcus garvieae*, are portrayed as one of those barriers and as a critical limiting factor in modern aquaculture. The antimicrobial properties of Lactic Acid Bacteria (LAB), mainly the production of organic acids and bacteriocins (*e.g.*, the lanthionine containing nisins A and Z; NisA and NisZ, respectively), led to propose LAB as probiotics to be used as an alternative and/or complementary strategy to vaccination and conventional chemotherapy, in aquaculture. *L. lactis* RBT18, isolated from cultured rainbow trout (*Oncorhynchus mykiss*, Walbaum), exerts a strong direct and extracellular antimicrobial activity against *L. garvieae* and other ichthyopathogens, being this antimicrobial activity heat-resistant (100ºC, 10 min), and thus suggesting the involvement of a thermostable antimicrobial compound (*i.e.*, bacteriocin). Cross-immunity tests using the agar-well-diffusion test (ADT) and PCR assays suggested that NisA/Z is the bacteriocin responsible for the extracellular antimicrobial activity exerted by *L. lactis* RBT18. To demonstrate this hypothesis, the bacteriocin was purified to homogeneity by two different multi-chromatographic procedures. MALDI-TOF/TOF MS analyses of purified samples after the last reverse-phase chromatography step identified, in both procedures, the presence of NisZ (3,330 Da), and its oxidized form (3,346 Da), derived from the oxidation of a lanthionine ring. Noteworthy, the oxidized form of NisZ showed a diminished antimicrobial activity, which could increase the chances of bacterial pathogens to evade its antimicrobial activity. Hence, the experimental approach carried out in this work constitutes an appropriate strategy for the preliminary identification of nisin-producing lactococcal strains and, in particular, both multi-chromatographic purification procedures were found to be suitable for the purification of NisZ, in both native and oxidized forms, with the purification procedure I being the most efficient and appropriate for this purpose, since the antimicrobial activity yield and the increase in specific antimicrobial activity were respectively 18 and 21-times higher than using the purification procedure II.

Further experiments are necessary to assess the *in vitro* and *in vivo* safety and efficiency of *L. lactis* RBT18 as probiotic in aquaculture but also to gain insight into the nisin oxidation process, and its implications on both bacteriocin pharmacokinetics and

pharmacodynamics, to optimize the environmental conditions leading to reduce bacteriocin oxidation and thus bacterial pathogen resistance.

Keywords: aquaculture; probiotics; lactic acid bacteria; antimicrobial activity; bacteriocins; purification; nisin Z.

RESUMO

Com o reconhecimento da aquacultura como o sector de produção alimentar que mundialmente regista o maior crescimento, novos obstáculos e desafios, derivados da sua inevitável intensificação, emergem. A esse respeito, as doenças infecciosas, como a lactococose causada por *Lactococcus garvieae*, não só são retratadas como um desses obstáculos, como também são um factor limitante crítico na aquacultura moderna.

As propriedades antimicrobianas apresentadas pelas Bactérias Ácido Lácticas (LAB), nomeadamente a produção de ácidos orgânicos e bacteriocinas (*e.g.* detentoras de lantionina como as nisina A e Z; NisA e NisZ respectivamente), levaram a que as LAB, em aquacultura, fossem propostas como probióticos de uso alternativo, e/ou complementar, às estratégias de vacinação ou quimioterapia convencionais. *L. lactis* RBT18, isolada a partir de uma produção de truta arco-íris (*Oncorhynchus mykiss*, Walbaum), demonstra uma forte actividade antimicrobiana directa e extracelular contra *L. garvieae* e outros ictiopatógenos, sendo esta resistente ao calor (100ºC, 10 min), sugerindo o envolvimento de um composto antimicrobiano termoestável (*i.e.*, bacteriocina). Testes de imunidade-cruzada, recorrendo ao *agar-well-diffusion test* (ADT), e testes PCR, sugeriram que a bacteriocina responsável pela actividade antimicrobiana extracelular demonstrada por *L. lactis* RBT18 seria a NisA/Z. De modo a demonstrar esta teoria, a bacteriocina foi purificada até à homogeneidade recorrendo a dois distintos protocolos multi-cromatográficos. Após as duas cromatografias, análises MALDI-TOF/TOF MS das amostras identificaram a presença de NisZ (3,330 Da), bem como da sua forma oxidada (3,346 Da), derivada da oxidação de um anel de lantionina. Notavelmente, a forma oxidada de NisZ apresentou uma actividade antimicrobiana diminuída, o que poderá aumentar as hipóteses de evasão ao efeito antimicrobiano por parte de determinados patógenos. Portanto, não só a abordagem desenvolvida ao longo deste trabalho demonstrou ser uma estratégia adequada para a identificação preliminar de estirpes productoras de nisina pertencentes ao género *Lactococcus*, como também ambos os protocolos de purificação multi-cromatográfica demonstraram ser apropriados para a purificação de NisZ, tanto na sua forma natural como oxidada. No entanto, o protocolo de purificação I demonstrou ser mais eficiente e adequado para o propósito, na medida em que o rendimento de actividade antimicrobiana e o aumento da actividade específica observados, foram respectivamente 18 e 21 vezes superiores aos verificados utilizando o protocolo de purificação II.

Não obstante, futuros estudos serão necessários para avaliar a segurança e eficiência, *in vitro* e *in vivo,* de *L. lactis* RBT18 como um potencial probiótico em aquacultura, bem como para obter um maior conhecimento sobre o processo de oxidação da nisina. De igual modo, será necessário avaliar as implicações da oxidação da nisina tanto na sua farmacocinética, como na sua farmacodinâmica, de modo a optimizar as condições ambientais que conduzem a uma menor oxidação da bacteriocina, portanto, reduzindo as resistências por parte de patógenos de origem bacteriana.

Palavras-chave: aquacultura; probióticos; bactérias ácido-lácticas; actividade antimicrobiana; bacteriocinas; purificação; nisina Z.

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LIST OF ABBREVIATURES

A Absorbance

ADT Agar-well-diffusion test

ARB Antibiotic Resistance Genes Database

ATP Adenosine triphosphate

AXOS Arabynoxylan-oligosaccharides

BC before Christ

BKD Bacterial kidney disease

BLAST Basic Local Alignment Search Tool

bp base of pairs

BU Bacteriocin unit

CARD Comprehensive Antibiotic Resistant database

CFS Cell-free supernatant

cfu colony-forming unit

CWD Cold water disease

Da Dalton

DGRM Direcção-Geral de Recursos Naturais, Segurança e Serviços Marítimos

DNA Deoxyribonucleic acid

EC European Commission

EDTA Ethylenediamine tetraacetic acid

EFSA European Food Safety Authority

ERM Enteric red-mouth disease

EU European Union

EUMOFA European Union Market Observatory for Fisheries and Aquaculture

EUR Euro

FAO Food and Agriculture Organization

FDA Food and Drug Administration

FOS Fructooligosaccharides

FPLC Fast-protein liquid chromatography

g gram

GOS Galactooligosaccharides

GRAS Generally Recognized as Safe

h hours

His Histidine

kDa kilo Dalton

kg kilogram

km kilometre

kV kilovolt

L litre

LAB Lactic acid bacteria

M molar

MALDI-TOF Matrix-Assisted Laser Desorption/Ionization-Time Of Flight

mBar millibar

mg milligram

min minutes

mL millilitre

mM millimolar

MOS Mannan-oligosaccharides

MPA Microtiter plate assay

MRL Maximum Residue Levels

mRNA Messenger RNA

MRS Man, Rogosa and Sharpe

MS Mass spectrometry

NACA Network of Aquaculture Centres in Asia-Pacific

NCBI National Centre for Biotechnology Information

nis Nisin gene

NisA Nisin A

NisZ Nisin Z

nm nanometre

PBL Planbureau voor de Leefomgeving (Netherlands Environmental Assessment Agency)

PCR Polymerase Chain Reaction

PMF Proton motor force

QPS Qualified Presumption of Safety

RNA Ribonucleic acid

RP Reversed-phase

RP-FPLC Reversed-phase fast-protein liquid chromatography

rpm revolutions *per* minute

rRNA Ribosomal RNA

RTFS Rainbow trout fry syndrome

SARS-CoV-2 Severe acute respiratory syndrome coronavirus 2

SCAN Scientific Committee on Animal Nutrition

sec seconds

SEGABALBP Grupo de Seguridad y Calidad de los Alimentos por Bacterias Lácticas, Bacteriocinas y Probióticos

SOAT Stab-on-agar test

t tonnes

TFA Trifluoroacetic acid

TY Tryptone Yeast Extract

UCM Universidad Complutense de Madrid

UK United Kingdom

UN United Nations

USA United States of America

USD United States Dollar

VFDB Virulence Factor DataBase

v/v volume/volume

WGS Whole genome sequencing

WHO World Health Organization

w/ with

w/v weight/volume

ºC degree Celsius

% percentage

µL microlitre

µm micrometre

1. INTRODUCTION

During the past few decades, aquaculture has been established as the fastest-growing food-producing sector worldwide. Moreover, aquaculture is regarded as a key alternative to supply the foreseeable human population growth and the increasing demand for food and protein sources, namely from aquatic origin (Defoirdt *et al.,* 2011; FAO, 2020).

The freshwater aquaculture systems have an increasing importance to the sector and represented the larger portion of the total production in 2018. In the European Union (EU), as well as in Portugal, this freshwater culture heavily relies on the production of the rainbow trout (*Oncorhynchus mykiss*), possessing a strong commercial interest (DGRM, 2014; EUMOFA, 2019; FAO, 2020; Pordata, 2020). Alongside the expansion and the required intensification of modern aquaculture, new challenges to the sector arose, namely the emergence of multiple ichthyopathogens that stand as a major limiting factor to the industry (Ringø *et al*., 2010; Pérez-Sánchez *et al*., 2014). In order to prevent and control the economic losses associated with infectious diseases, such as the bacterial ones, the indiscriminate use of veterinary drugs was, to some extent, a common procedure. The widespread use of antibiotics in aquaculture has been associated with harmful effects for human, animal, and public health, with the emergence of antibiotic resistances. Subsequently, multiple countries have established strict regulations concerning the use of antibiotics in aquaculture, which include measures such as the prohibition of the use of antibiotics as prophylactic agents, the reduction of authorized substances, the establishment of maximum residue limits, amongst others (Guardabassi *et al.,* 2000; Cabello, 2006; EFSA, 2008a, 2008b; Ringø *et al*., 2014). Under these circumstances, alternatives are required to control and prevent the existing and emerging infectious outbreaks in the sector, and to substitute the traditional chemotherapy practices. These alternatives include practices such as vaccination, the use of immunostimulants and the use of probiotics. In this respect, probiotics, which are defined as live microbial adjuncts that have beneficial effects on the host through different proposed mechanisms, pose as promising and effective alternatives or complementary strategies to the conventional chemotherapy (Verschuere *et al.,* 2000a; Balcázar *et al.*, 2006; Merrifield *et al.,* 2010a; Defoirdt *et al.,* 2011; Pérez-Sánchez *et al.*, 2014, Gómez-Sala *et al.*, 2019).

Concerning the use of probiotics in aquaculture, lactic acid bacteria (LAB), a large and diverse group of Gram-positive bacteria that include, for instance, the genera *Lactococcus, Lactobacillus, Leuconostoc, Enterococcus, Pediococcus,* attract for various

reasons a great amount of interest and potential usages. Not only are LAB generally regarded as safe microorganisms for human and animal consumption, but also, some LAB have already been proposed, and in some circumstances accepted, as probiotics in human medicine and animal production. Likewise, the main LAB so far proposed as probiotics in aquaculture have shown promising results and beneficial effects on the hosts, such as increasing the survival rates during infectious outbreaks, modulating the immune system, improving the water quality, or growth stimulation (Verschuere *et al.,* 2000a; Nayak, 2010; Nishie *et al.,* 2012; Pérez-Sánchez *et al.,* 2014; Gómez-Sala *et al.,* 2019).

There are several mechanisms by which LAB exert their probiotic action, out of which, their antimicrobial properties stand as a crucial one. The most prominent antimicrobial properties of LAB include the competition for nutrients, production of organic acids (namely lactic acid), as well as, the production of antimicrobial compounds, such as the ribosomally-synthetized antimicrobial peptides known as bacteriocins (Cintas *et al.,* 2001; Cotter *et al.,* 2005; Nishie *et al.,* 2012; Gómez-Sala *et al.,* 2019).

Bacteriocins comprise a vast and heterogenous group of proteinaceous compounds synthetized by some bacteria, mainly Gram-positive ones, with wide applications that can range from food technology to probiotic and therapeutic usages. Concerning the variety of bacteriocins so far discovered, nisin, a bacteriocin that belongs to the lantibiotic class, stands probably as the most well-studied one. Furthermore, nisin is to date the only bacteriocin with a legal use approved in the EU, as a food additive. Nevertheless, nisin is a bacteriocin with a broad spectrum of antibacterial activity, and therefore, it also has a strong pharmaceutical and therapeutical potential (Guder *et al.,* 2000; Cintas *et al.,* 2001; 2011; Cotter *et al.,* 2005; Zendo *et al.,* 2010; Nishie *et al.,* 2012).

Whether obtaining a bacteriocin for experimental or commercial purposes, the purification procedure is in both cases a critical and diverse step for their characterization (Guyonet *et al.,* 2000; Cintas *et al.,* 2001; Saavedra *et al.,* 2004).

Thus, this experiment, performed in the Grupo de Seguridad y Calidad de los Alimentos por Bacterias Lácticas, Bacteriocinas y Probióticos (SEGABALBP), Sección Departamental de Nutrición y Ciencia de los Alimentos (Nutrición, Bromatología, Higiene y Seguridad Alimentaria), Facultad de Veterinaria, Universidad Complutense de Madrid (UCM), aimed to perform an appropriate and effective preliminary identification of a bacteriocinogenic strain with a potential probiotic use in aquaculture. Hence, the antimicrobial properties and activity of a bacterial strain previously isolated from cultured rainbow trout and subsequently stored, were therefore assessed. Then, two different

multi-chromatographic procedures were performed in order to purify to homogeneity a putative bacteriocin produced by the isolated strain (RBT18).

2. LITERATURE REVIEW

2.1. Definition, background, and economic importance

Currently, the projection of the United Nations (UN), dated 2015, is that by 2050 the world population will reach an astonishing 9.5 billion people. This worldwide population growth, along with other important factors will inevitably lead to a higher demand for food supply and for protein sources. These factors include globalisation, increased income of the general population, globalised urbanisation and, due to better health care systems, an ageing population. It is also projected that due to this human population growth, especially in developing countries, the socio-economic changes that will occur will double the current demand for protein sources by 2050. Fish is one of the main sources of protein in human diet, in 2018 the world *per capita* consumption of fish was estimated around 20.5 kg, which constitutes a new record peak. In 2015 it is estimated that 17% of the total protein consumption of the world population was obtained from fish sources. The consumption of fish is also a key factor in what is considered to be a healthy diet. Fish not only has a higher protein content compared to other standard animal protein sources (for instance, meat) but also has a lower feed conversion rate. This fish protein is also more digestible and richer in the so-called essential amino acids, such as methionine and lysine. Other health benefits can also be related to the abundant presence of long-chain polyunsaturated fatty acids in fish and shellfish. Regular consumption of fish is associated with health benefits such as decreasing the risk of heart, mental and bone diseases, controlling blood pressure and inflammation and preventing arthritis. Fish is also a rich source of multiple minerals and vitamins and therefore constitutes an essential part of the human diet, with a considerate nutritional value (PBL, 2011; Abedi and Sahari, 2014; Sampels, 2014; UN, 2015; Henchion *et al*., 2017; FAO, 2020).

The fish supply can be provided either by marine and inland fisheries or by aquaculture systems. According to the Food and Agriculture Organization of the UN (FAO), aquaculture can be defined as: “farming of aquatic organisms including fish, molluscs, crustaceans and aquatic plants”. This concept includes rearing aquatic organisms both under controlled or semi-controlled conditions: “farming implies some sort of intervention in the rearing process to enhance production, such as regular stocking, feeding, and protection from predators. Farming also implies individual or corporate ownership of the stock being cultivated. For statistical purposes, aquatic organisms which

are harvested by an individual or corporate body which has owned them throughout their rearing period contribute to aquaculture while aquatic organisms which are exploitable by the public as a common property resource, with or without appropriate licences, are the harvest of fisheries" (FAO/NACA/WHO, 1999).

The worldwide fish production, which includes fisheries and aquaculture, reached its peak in 2018 with 179 million tonnes (t) (*Figure 2.1*). Out of the total, aquatic animals' aquaculture represented 82 million t, which included: 54.3 million t of finfish, 17.7 million t of molluscs, 9.4 million t of crustaceans and 936,700 t of other aquatic animals such as turtles, frogs and edible jellyfish. The first sale value of those 179 million t combined in 2018 was estimated in 401 billion United States Dollar (USD). Of which, around 250 billion USD were revenue from aquaculture activity, which represents around 62% of the total economical outcome. The global fish consumption continues to grow and in fact it has outpaced the human population growth rate. Between 1961 and 2017 the human population growth was 1.6% and the fish consumption growth was 3.1%. Furthermore, fish consumption has also outpaced global meat consumption growth, which was 2.7% within the same period. This rate increase can be once again exemplified by comparing the global *per capita* consumption in 1961, which was 9 kg, with the 2016 global *per capita* consumption: 20.5 kg. The expanding role that aquaculture represents in the *per capita* consumption of fish is exposed in *Figure 2.2*. If in 1958 captured fish had a major role in consumption *per capita*, the balance of consumption has now changed. In 2013 aquaculture surpassed for the first time the wild-caught fish share of consumption. In 2018 the aquaculture share was estimated in 52% of the total consumption *per capita*. By 2030, this number is expected to reach a share of 59% (FAO, 2018; 2020).

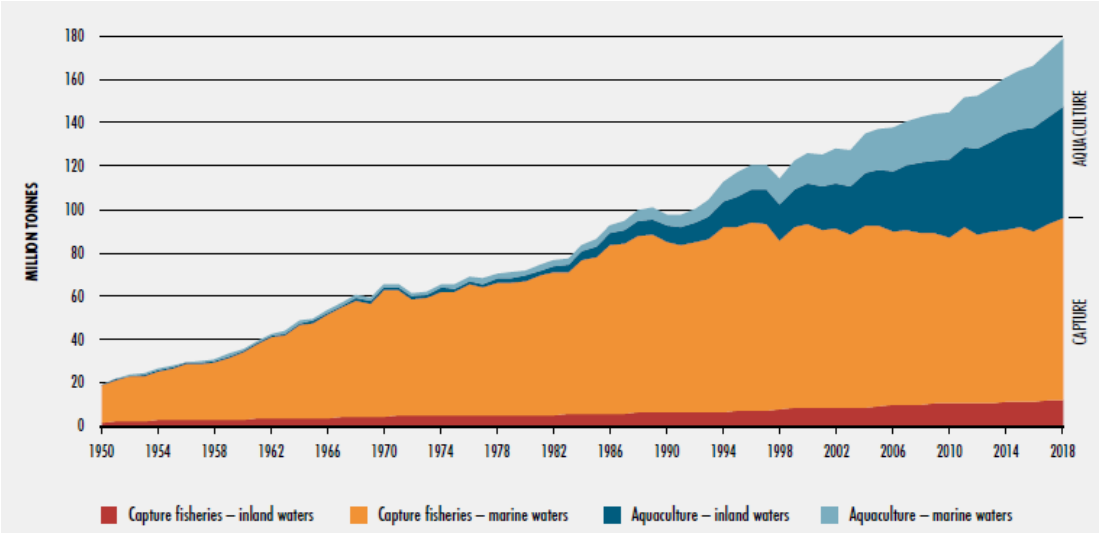

Figure 2.1. World capture fisheries and aquaculture production between 1950-2018 (millions of tonnes).
Source: FAO (2020).

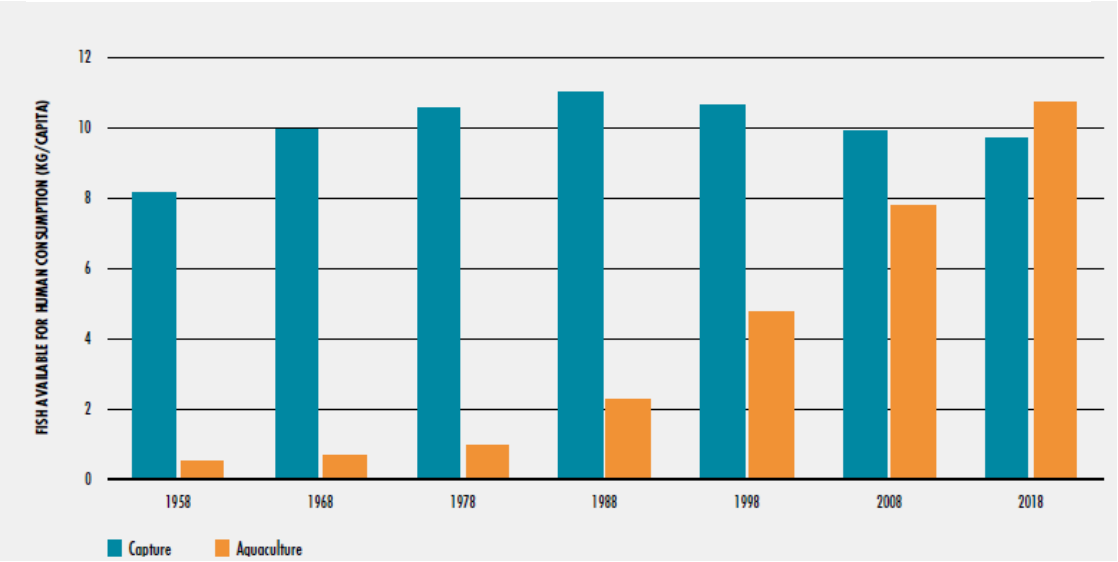

Figure 2.2. Contribution of fisheries and aquaculture to fish consumption (kg/*capita*) between 1958-2018.
Source: FAO (2020).

Since the beginning of the new millennium aquaculture no longer experiences the annual growths observed in the past (in the 1980s it was 10.8% and in the 1990s it was 9.5%). However, if this is true, then it is also true that the aquaculture sector is still the fastest growing food production sector. Despite everything the annual growth between 2001 and 2018 was still 4.5% (FAO, 2020).

Apart from being a cornerstone in the food supply chain all over the world, fisheries and aquaculture also represent a major role in the socio-economic lives of millions of people around the world. The fish production sector is the livelihood and source of economical sustainability for millions. The FAO State of the World Fisheries and

Aquaculture report from 2018 indicates that the sector employs around 59.5 million people all over the world, from which around 20.5 million are engaged in the aquaculture activity. The importance of aquaculture for the entire sector is rising since its proportion doubled from 17% in 1990 to 34% in 2018. On the opposite side, fisheries had a decrease in their proportional importance decreasing from 83% in 1990 to 66% in 2018 (FAO, 2020). It is also important to state that China has a major role since it represents around 25% of all the work force of the sector worldwide. In 2016, in China, 9.4 million people were involved in fisheries and around 5 million people in aquaculture (FAO, 2018).

2.1.1. Global aquaculture production

In the analysis of the world's farmed fish production, the importance of China to the sector is very well stated. Although there has been a decrease in its proportional representation of the world´s total aquaculture production, China´s aquaculture production represented around 57.9% of the world total in 2018, which still holds great importance. This importance can be called "the China factor". China itself has produced more farmed fish than the rest of the world combined since 1991. Chinese aquaculture represents around 73.7% of the country's total fish consumption which has proved itself as an essential contribution in feeding the impressive Chinese population. *Table II.1* shows the aquaculture food fish production by regions and by major producers. From *Table II.1* it is also possible to conclude that, despite Europe's higher production, its proportional share is decreasing from 6.5% to 3.75% of the world's total. The same trend can be observed in North America (from 1.96% in 1995 to 0.80% in 2018). On the other hand, regions where developing countries are emerging, such as Africa and Asia for instance, aquaculture's share is rising. This rise could be explained by the demographic and socio-economical changes that are occurring there. A higher population growth as well as an increasing economically active population in the primary sector could already well be decisive factors in the present and become even more influent on the future (FAO, 2018; 2020).

Table II.1. Aquaculture production (thousands of tonnes and share) by region or country, between the years of 1995-2018.

Region/Country	1995	2000	2005	2010	2015	2018
Africa	110.2 0.45%	399.6 1.23%	646.4 1.46%	1 285.8 2.23%	1 777.6 2.44%	2 195.9 2.67%
Latin America/Caribbean	440.9 1.81%	838.9 2.59%	1 508.4 3.40%	1 855.6 3.21%	2 661.3 3.66%	3 139.6 3.83%
North America	478.7 1.96%	584.5 1.80%	668.5 1.51%	659 1.14%	613.4 0.84%	659.6 0.8%
Asia (w/ China)	5 811.4 23.87%	6 898.5 21.28%	11 083.2 24.98%	15 715.4 27.22%	20 843.6 28.64%	25 253.1 30.76%
China	15 855.7 65.03%	21 522.1 66.39%	28 120.7 63.48%	35 513.4 61.50%	43 748.2 60.12%	47 559.1 57.93%
Oceania	94.2 0.39%	121.5 0.37%	151.5 0.34%	187.8 0.33%	178.5 0.25%	205.3 0.25%
Europe	1 581.4 6.49%	2 052.6 6.33%	2 137.3 4.82%	2 527.0 4.38%	2 948.6 4.05%	3 082.6 3.75%
World	**24 382.5**	**32 417.7**	**44 298.0**	**57 743.9**	**72 771.3**	**82 095.1**

Source: FAO (2018).

2.1.2. Types of aquaculture systems

There are several different aquaculture methods and practice systems. Worldwide, a great variety of organisms are farmed in different environments, including freshwater, brackish water, and seawater. It can also range from extensive to intensive systems, depending on the level of controlled conditions and management input imposed. Extensive systems consist in low rearing density and with the minimum, or even none, artificial feeding input. Intensive systems consist in high density farming with total food input associated (Araújo, 2015).

2.1.2.1. Freshwater aquaculture

The inland aquaculture is an increasingly important form of aquaculture. It is mainly practiced under freshwater conditions. In 2018, out of 82 million tonnes of food fish production, around 51.3 million tonnes (around 62.5% of the total production) were the product of inland aquaculture. The main product of inland aquaculture is, without doubt, finfish. Inland finfish production in 2018 was around 47 million tonnes, which approximately constitutes 91.5% of the total freshwater aquaculture production (*Table II.2*) (FAO, 2020).

The extensive freshwater aquaculture system is characterized by the existence of ponds. These ponds allow the fauna to reach levels of development otherwise impossible under natural conditions. Finfish are the most representative group of species farmed under these conditions. A common family of species being farmed this way is the carp-family (*Cyprinidae*) (Araújo, 2015; FAO, 2020).

In the intensive freshwater systems, fish are raised in many types of tanks. These tanks can vary in dimensions and depths, but they are all projected to potentiate the most growth-stage of the specie involved until it reaches marketable size. There are two different techniques in this intensive method: continuous flow (the water enters upstream and leaves the tank downstream, also known as flow-through system) and recirculation (it is a closed system circuit where the water is recycled and reused in the tanks). The recirculation system is more expensive and costly, not only because of the energy costs, but also because it requires complex technologies. However, this method can separate the tanks from the natural environment, making it possible to control water parameters such as temperature, acidity, salinity, amongst others, hence enhancing its quality and maximizing the fish development. This method is used for instance to farm rainbow trout (*Oncorhynchus mykiss*), eel (*Anguilla anguilla*), catfish (order Siluriformes) among many others (Araújo, 2015).

2.1.2.2. Brackish water aquaculture

This system is practised in completely or partially human-made structures, most of the time resembling lagoons or tanks, in shore-based or coastal areas. It can be practiced either under extensive or semi-extensive conditions. Sometimes it involves the introduction of fries from hatcheries or feed supply, which therefore establishes a semi-

extensive system. In the natural shored-base structures salinity is not stable as in other aquaculture systems. Due to environmental conditions such as rainfall intensity, evaporation or even on the season of the year, this stability is not granted. Species commonly farmed under these conditions are eel, common sole (*Solea solea*), Senegalese sole (*Solea senegalensis*), shrimps (infraorder *Caridea*), amongst others (Araújo, 2015; FAO, 2020).

2.1.2.3. Marine aquaculture

Marine aquaculture can also be known as mariculture. It is practiced in a marine water environment, either in the sea or in coastal facilities. In 2018, the food fish production of marine aquaculture and coastal aquaculture combined was estimated around 30.8 million tonnes. From which, around 17.3 million tonnes were shelled molluscs (around 56% of the total marine aquaculture production), 7.3 million tonnes were finfish and 5.7 million tonnes were crustaceans, representing together approximately 42.2% of the total production (*Table II.2*) (FAO, 2020).

Marine fish, especially flatfish, are commonly farmed in coastal infrastructures. They are usually human-made shore-based tanks. These tanks are supplied with seawater that gets pumped directly from the sea and then into recirculation. The water recirculation grants a more controlled environment, which is fit to obtain optimal production parameters in hatcheries and nurseries. Flatfish such as common sole, Senegalese sole or turbot (*Scophthalmus maximus*) are regularly farmed in this system (Araújo, 2015).

Another common technique consists of the use of marine cages. The fish are held captive in these structures, which are anchored to the bottom of the sea and are kept floating at surface, through means of multiple types of devices. This kind of technique is used in sheltered zones near shore, where for instance Atlantic salmon (*Salmo salar*) is usually bred (Araújo, 2015).

2.1.2.4. Shellfish farming

Among the most common species of shellfish bred are oysters (*Ostrea* spp.), mussels (family *Mytilidae*), clams (orders *Myoida* and *Veneroida*) and abalones (*Haliotis* spp.). Usually the shellfish farming systems consist of the collection of wild specimens or hatcheries placed in strategic places, with no human input, meaning that nutrition must

be natural and provided by the environment. Multiple techniques for shellfish farming have been developed throughout the years. They can be gathered in two big groups: bottom-farming and off-bottom farming, such as suspended long-lines, raft methods or hanging systems (Chinabut *et al*., 2006; Araújo, 2015).

Table II.2. Aquaculture production in thousands of tonnes in the year of 2018.

Category	Inland aquaculture	Marine aquaculture	Total production (2018)
Finfish	46 951	7 328	54 279
Crustaceans	3 653	5 734	9 387
Molluscs	207	17 304	17 511
Other aquatic animals	528	390	919
Total	**51 339**	**30 756**	**82 095**

Source: FAO (2020).

2.1.3. Species produced in aquaculture

In terms of species, the report of FAO demonstrates that in 2018 there were 622 different species farmed worldwide. Of these 622 species, 466 were considered individual species, 7 interspecific hybrids of finfish, 92 species groups at genus level, 32 at family level and 25 at level of order or even higher. In 2006, twelve years before the results consulted in the 2020 FAO's report, the number of species farmed was 472. This represents an increase of 31.8% in just twelve years. Although there is a huge variety and an increasing number of species farmed worldwide, aquaculture is dominated by a small group of species. For instance, 90% of the finfish sector was dominated by 27 species, and the 20 most produced species were responsible for around 84% of it. In comparison with the finfish sector, molluscs, crustaceans, and other aquatic animals share less diversity among them (FAO, 2020).

In 2018, out of the 54 million tonnes of finfish produced, more than 18 million tonnes derived from the five most common carp species farmed. These five carp species represent around 34% of the total finfish production of 2018, which states the dominance and importance of the carps in world production. Tilapias (mainly Nile tilapia, *Oreochromis niloticus*) also have a significant weight on the world's balance of production, representing more than 4.5 million tonnes in 2018, being mainly raised in Asia and Africa. Another important group of fish to point out is that of the diadromous

fish, the most farmed one was the Atlantic salmon (around 2.4 million tonnes in 2018), followed by the milkfish (*Chanos chanos*, around 1.3 million tonnes in 2018), and then by the rainbow trout (with around 848 000 tonnes in 2018) (Araújo, 2015; FAO, 2020).

2.1.4. The production by the major producers

The world leading aquaculture producers have many differences among them. Thus, the group of species farmed as well as the aquaculture systems itself vary significantly among them. As stated above, China is the dominant factor in the world's aquaculture production. China alone produces around 47.5 million tonnes (excluding aquatic plants production), representing around 58% of the world's total production. The rest of the world combined, excluding China, produces around 34.5 million tonnes (excluding aquatic plants). All five of the production leading countries belong to the Asian continent: China, India, Indonesia, Vietnam, and Bangladesh (ordered from the top producer to the least) (FAO, 2020).

Concerning the inland finfish production, the sector is once again dominated by the same leading Asian countries. In addition, other important inland production countries like Egypt or Brazil, rely almost all of their total production in inland farming systems. On the other hand, being the only European country among the world's major producers, Norway relies its production on mariculture, more precisely in the marine cage system, mostly producing Atlantic salmon and making Norway the second biggest finfish producer from marine or coastal aquaculture. In the marine or coastal production Norway competes side by side with China. Indonesia is also considered a major producer in this sector; its production relies heavily on coastal brackish water ponds (Araújo, 2015; FAO, 2020).

The marine crustacean production, which excludes the marine shrimp species that are produced in inland systems, is also dominated by Asia, with some South and Latin American countries entering the race, like Ecuador, Mexico, and Brazil. Countries like Republic of Korea (South Korea) or Japan rely a large portion of their production on the marine molluscs' production. Alongside with China and Chile, they are the big major producers. But once again, China leads by far in the production rankings, producing 14.4 million tonnes of marine molluscs, which represents almost 82% of the sector. China has managed to greatly diversify its aquaculture sector, either in terms of species produced, or in terms of aquaculture systems used, as demonstrated before. Its finfish culture in

freshwater systems has a major role providing food fish for its domestic and internal growing market (Araújo, 2015; FAO, 2020).

2.1.5. Aquaculture in the European Union

In 2017, the EU *per capita* consumption of fish was slightly higher than the world's average, being 24.35 kg. Nevertheless, this represents a small decrease in comparison with the 2016 *per capita* consumption, which was 24.87 kg. Despite the EU's fish and shellfish consumption being higher than the world's average, its main source of fish supply differs. Around three quarters of that consumption in the EU is covered by wild-caught fisheries, contrasting with an increasing importance of aquaculture in the world's *per capita* consumption, which represented 53% in 2016. The aquaculture products *per capita* consumption, in 2017, was only estimated in 6.35 kg, which still represents an increase of 2% over the last decade (EUMOFA, 2019).

According to "the EU Fish Market" 2019 report, from the European Market Observatory for Fisheries and Aquaculture Products (EUMOFA), in 2017 the aquaculture production in the EU was estimated around 1.37 million tonnes, valued at 5.06 billion Euro (EUR). This represents an increase of 5% in production compared with the previous year, 2016, and a significant increase of 15% in the market value. The market value has almost doubled since 2008 (47%), while the production has increased 11% during the same period. Some of these economic changes can be traced back to factors such as the increasing production of high value species, such as the Atlantic salmon, seabass (*Dicentrarchus labrax*) or bluefin tuna (*Thunnus thynnus*), combined with a price increase in some of the major traded species, as well as a higher demand for quality food fish products. In the EU, bivalves, mussels, and other aquatic invertebrates still stand for almost half of the total production, despite a slight decrease in its importance share. The salmonids, like the Atlantic salmon or the rainbow trout, represent the second biggest group of the EU aquaculture production. These salmonids are the strongest economic group, representing 40% of the total farming value, in EUR (EUMOFA, 2019).

Table II.3. Volume (thousands of tonnes) of Aquaculture Production in the EU five bigger producers.

Country	2008	2016	2017	2008-2017	% of EU production (2017)
Spain	252	287	315	+25%	**23%**
UK	180	194	222	+24%	**16%**
France	238	182	189	-20%	**14%**
Italy	158	157	156	-1%	**11%**
Greece	94	123	126	+34%	**9%**
Top Five EU Producers	**922**	**943**	**1 008**	**+9%**	**73%**

Source: EUMOFA (2019).

The most important aquaculture producers in the EU, in 2017, considering that when the data was gathered the United Kingdom (UK) was still part of the EU, are by order: Spain, UK, France, Italy, and Greece. European aquaculture is characterized by a high level of production specialization. For instance, Greece has a production focused on gilt-head seabream (*Sparus aurata*) and seabass, Spain on mussels (family *Mytilidae*), especially in Galicia, and turbot. Furthermore, France on oyster production (which represents almost half of the countries' total production), Italy on clam and rainbow trout and the UK on Atlantic salmon. These five major producers are extremely important to the Union's production. In 2017, almost 75% of both total production and value in the EU (*Table II.3*) came from these five countries (EUMOFA, 2019).

2.1.6. Aquaculture in Portugal

Portugal leads by far the *per capita* fish consumption among the Member States of the EU. In 2017, the Portuguese *per capita* fish consumption was estimated in 56.8 kg, this is more than double of the other Member States average, which was in 2017, as stated above, 24.3 kg (EUMOFA, 2019).

The coastal extension of the Portuguese mainland has a length of 1,187 km. Across those kilometres it is possible to observe the existence of some firths and estuaries, which could gather conditions for the practice of aquaculture. Despite this, the weather and sea conditions, especially throughout the winter, are not considered as optimal, being a coastal shore very exposed to climate conditions. Nevertheless, the southern shore of the mainland, the island of Madeira, as well as some estuaries, lagoons, and bays gather

enough conditions to establish the practice of aquaculture. All these factors combined led to the emergence of technological solutions and resources, allowing the practice of aquaculture both in coastal areas and inland (DGRM, 2014).

In the decade of 1970, the Portuguese aquaculture production was dominated by species like the mullet (family *Mugilidae*), which at some point represented almost 80% of the national production. Throughout the decade of 1980, the inland aquaculture experienced a great development, especially in the farming of rainbow trout and some bivalves, like the clam (order *Myoida*) in brackish and seawater. The last decade of the century, the 1990s, is characterized by a strong development of mariculture. Initially it was focused on species like the seabass or the gilt-head seabream. However, more recently, species like the turbot, have experienced an interesting growth. Nowadays, the most important species farmed in Portugal can be separated, considering the type of water environment. The inland freshwater production relies almost exclusively on the rainbow trout, while the mariculture is dominated by the farming of gilt-head seabream, seabass and turbot, some of which, as a flat-fish, are now starting to be produced in coastal systems with water recirculation. The production of molluscs, mainly bivalves and mussels, has been rising considerably, holding nowadays a very significant role and share in the national production (around 67%). (*Table II.4*) (DGRM, 2014; Pordata, 2020).

Table II.4. Comparison of the main fish species farmed in Portugal between 2008 and 2018.

Main fish species farmed in Portugal (tonnes and %)						
Year	**TOTAL**	**Freshwater**	**Mariculture**			**Molluscs**
		Rainbow Trout	Turbot	Gilt-head seabream	Seabass	
2008	7 987	941 (11.78%)	351 (4.44%)	1 635 (20.47%)	1 069 (13.38%)	3 912 (48.97%)
2018	13 992 (+75.18%)	665 (4.75%)	2 582 (18.45%)	898 (6.41%)	200 (1.42%)	9 382 (67.05%)

Source: Pordata (2020).

2.2. Challenges and problems for the aquaculture sector

The state of fisheries resources is progressively deteriorating worldwide, due to factors such as a growing demand for fish consumption, lack of new fishing areas, and over-exploitation of the existent marine resources. While back in 1974, 90% of the marine fish stocks were fished within biologically sustainable parameters, in 2017 that number decreased to around 65.8%. Furthermore, fish stocks fished at unsustainable levels have increased from 10% in 1974 to around 34% in 2017. It constitutes a major challenge for

the world to rebuild those 33% of fish stocks that are currently overfished. Rebuilding requires worldwide commitment and, especially, time, since it usually takes two to three times the species' life span to achieve it. Aquaculture, therefore, is considered a strong and decisive strategy to meet both the growing human demand for fish and the ecosystem crisis that marine fish stocks face (FAO, 2020).

Aquaculture has expanded, diversified, intensified, and been modernized over the last few decades. Nevertheless, during this progress aquaculture has often struggled with conflicting objectives. For instance, biological criteria, such as growth rate, survival rate, food conversion efficiency or seed production, are of the utmost importance and are essential to achieve acceptable production parameters. Nonetheless, so are the objectives of reducing economical costs, maintaining water quality, preventing disease outbreaks, and minimizing economical and market exposure. A common difficulty opposing biological and economic criteria, might be that of opposing growth rate and maximum production. For instance, to obtain maximum growth it would involve conditions such as low stock density, which may not correspond to the maximum production. On the other hand, and to maximize production and profit, some farmers have been working under overproduction standards. This overproduction involves for instance, high stock density, which combined with other factors, such as temperature and salinity changes, or human handling, might work as stress factors and may lead to an increasing vulnerability of the fish to multiple pathogens and other opportunistic microorganisms, which can eventually result in high mortality rates and therefore, economic losses (Webber and Riordan, 1976; Cuenco, 1989; Brock and Bullis, 2001; Toranzo *et al.*, 2005; Araújo, 2015;).

Infectious disease outbreaks, especially those affecting larvae and alevin stages, represent a major limiting factor to the success, development, and expansion of aquaculture. Not only does the surrounding environment of aquatic farmed animals has the ideal conditions of growth for a huge variety of pathogenic microorganisms, but additionally, fish can also act as natural reservoirs of a variety of microorganisms. Some of them, can be highly pathogenic, whilst others might also be zoonotic. Both environmental and indigenous microflora, can pose important threats to human and public health and to food safety. Other microorganisms, like *Salmonella* sp. or *Escherichia coli*, can also be considered a threat through environmental contamination, mainly by primary sector and human waste. The combined effect of stress factors, the inevitable pathogen exchange between wild fish, farmed fish and even between different ponds, cages and farms, in addition to the environmental natural conditions, and its contamination, provides

the perfect scenario for the emergence and establishment of disease outbreaks (Murray and Peeler, 2005; Almeida *et al.*, 2009; Ringø *et al.*, 2010; Haenen *et al.*, 2013; Pérez-Sánchez *et al*., 2014; Araújo *et al*., 2015a).

2.2.1. Infectious diseases in fish

Fish diseases can be caused by a large variety of biological agents, such as bacteria, virus, parasites, and to a lesser extent, fungi, However, bacterial diseases represent the biggest share and the main challenge in the growing industry of aquaculture. Even so, viral diseases still hold some importance and can generate heavy economic losses. However, they do not seem to represent a threat to human health, since they are generally considered not to be pathogenic to humans. There are multiple viral families broadly studied in species with commercial value, like Iridovirus, Rhabdovirus, Reovirus, Herpesvirus, and Orthomyxovirus. Some of the most important include two rhabdoviruses that cause two different and important infectious diseases in salmonids: infectious haematopoietic necrosis and haemorrhagic septicaemia. Additionally, one orthomyxovirus is responsible for the infectious salmon anaemia, that was first reported in 1984 in Norway, but which is currently spread worldwide. Parasites, like some helminths, can pose a hazard on human health and food safety, especially in countries with cultural habits of consumption of raw or undercooked fish. Salmonids can also act as reservoirs for sea lice (family *Caligidae*), which can easily be spread among farmed fish. Moreover, parasite infections can cause local secondary bacterial infections on damaged tissues (Murray and Peeler, 2005; Almeida *et al.*, 2009).

2.2.2. Bacterial fish diseases

Bacterial fish diseases represent a major threat to the aquaculture sector. There are many bacteria involved in infectious diseases in fish, some of them associated with important economic losses. Both Gram-positive and Gram-negative bacteria can be involved in fish diseases. Thus, the main bacterial diseases involving mariculture and inland aquaculture are: i) Gram-positive such as *Carnobacterium* spp. (*Carnobacterium maltaromaticum*), *Lactococcus* spp. (*Lactococcus garvieae* and *Lactococcus piscium*), *Mycobacteriaceae* (*Mycobacterium marinum*), *Nocardiaceae*, *Piscirickettsia* (*Piscirickettsia salmonis*), *Renibacterium* spp. (*Renibacterium salmoninarum*),

Streptococcus spp. (*Streptococcus iniae*, *Streptococcus parauberis*, *Streptococcus phocae*, *Streptococcus agalactiae*), *Vagococcus* spp. (*Vagococcus salmoninarum*); ii) Gram-negative bacteria such as *Aeromonadaceae* like *Aeromonas* spp. (*Aeromonas salmonicida* and *Aeromonas hydrophila*), *Aliivibrio* spp. (*Aliivibrio salmonicida*), *Enterobacteriaceae* such as *Yersinia* spp. (*Yersinia ruckeri*) and *Edwardsiella* spp. (*Edwardsiella tarda*), *Flavobacterium* spp. (*Flavobacterium psychrophilum* and *Flavobacterium columnare*), *Listonella pelagia, Photobacterium* spp. (*Photobacterium damselae*), *Pseudomonas* spp. (*Pseudomonas anguilliseptica* and *Pseudomonas fluorescens*), *Tenacibaculum* spp. (*Tenacibaculum maritimum*) and multiple *Vibrionaceae* like *Vibrio* spp. (*Vibrio alginolyticus*, *Vibrio (Listonella) anguillarum*, *Vibrio campbellii*, *Vibrio harveyi*, *Vibrio parahaemolyticus*, *Vibrio splendidus* and *Vibrio vulnificus*) (Fryer and Hedrick, 2003; Ghittino *et al*., 2003; Blanco *et al.*, 2004; Toranzo *et al.*, 2005; Araújo, 2015).

2.2.2.1. Most important bacterial fish diseases in rainbow trout production

Rainbow trout represents a key role in the world's freshwater aquaculture production, which was 848 000 tonnes in 2018. The rainbow trout production was also an essential part of this work and of its objectives. In the rainbow trout production, infectious diseases also represent a major obstacle to the production standards. Infectious diseases can represent up to 10% of the total losses of fish during the farming process. Some of these infectious diseases, combined and amplified by other factors (like stress factors), can present a mortality rate up to 90%. Considering the bacterial diseases in the rainbow trout production, lactococcosis represents around 45% of the total, which stands for the biggest share. Lactococcosis is followed by yersiniosis (13.6%), furunculosis (11%), streptococcosis (7.3%), vibriosis (2.5%), and others (20.6%) (*Figure 2.3*) (Blanco *et al.*, 2004; FAO, 2020).

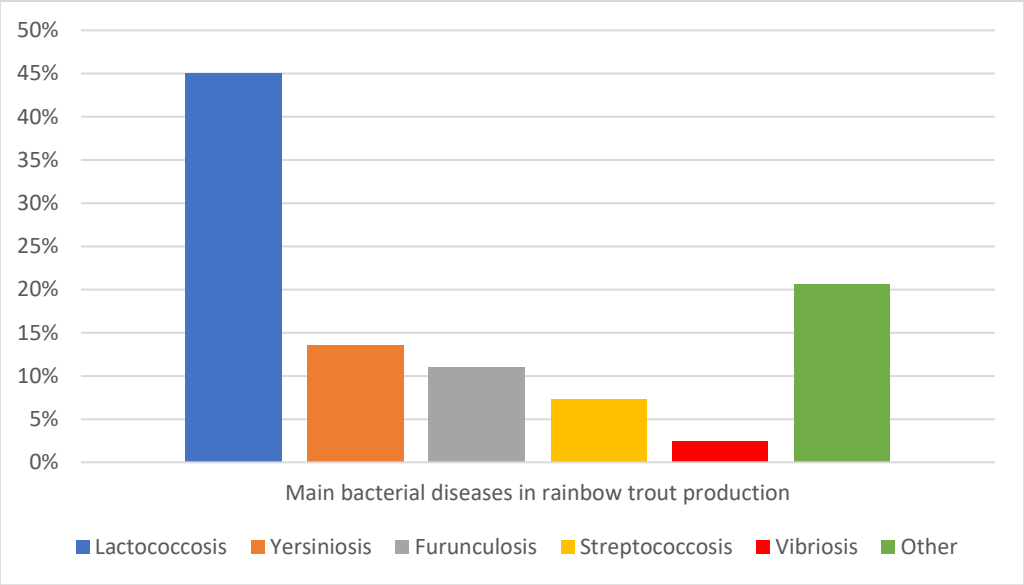

Figure 2.3. Main bacterial diseases in rainbow trout production, in percentage.
Adapted from: Blanco et al. (2004).

2.2.2.2. Lactococcosis

The bacterial agent behind lactococcosis is a Gram-positive coccus called *Lactococcus garvieae*. The disease, formerly known as streptococcosis, was first reported in fish during the decade of 1950 in Japan, in an intensive rainbow trout production farm. In 1985, the bacterial agent was for the first time isolated, in Great Britain, from a cow's mastitic udder, being by that time classified as *Streptococcus garvieae* (Aguirre and Collins, 1993). Since then, the disease was reported worldwide, affecting multiple species, among them the rainbow trout. The losses associated with lactococcosis can range from 50 to 80%. There are multiple hosts for the bacterial agent, among them humans, suggesting its potential as an emerging zoonotic agent, being sometimes linked with aquaculture-related outbreaks. The introduction of new lots of fish in the farm, of which some might be asymptomatic carriers, and the spreading of bacteria through faeces to other fish, are considered the main infection source in a farm. Moreover, some fish that recovered from lactococcosis have reportedly continued to disseminate the agent during some period (Vendrell *et al*., 2006; 2008; Chan *et al*., 2011).

The disease is characterized by a hyperacute haemorrhagic septicaemia and meningoencephalitis. Its evolution might depend on water conditions, such as temperature. In this respect, lactococcosis has been linked with summer months and with higher water temperatures (usually above 18ºC, but also described before in temperatures

between 14-15ºC), and with poor sanitary conditions of the water. The incubation period tends to be short (around 2-3 days), with a rapid and general anorexia, melanosis, lethargy, loss of orientation and erratic swimming. External clinical findings are often exophthalmia (uni- or bilateral), haemorrhages in the periorbital and intraocular area, the base of fins, the perianal region, the opercula, and the buccal region. Anal prolapses can often be found too. Furthermore, the vascular endothelium is damaged, which causes lesions in the most irrigated tissues. During necropsy, it is usually found liquid in the peritoneal cavity, which can be purulent or haemorrhagic. Macroscopic lesions can vary from strong congestion in internal organs, different levels of haemorrhages in the swim bladder, intestine, liver, peritoneum, spleen, and kidney. Other lesions like focal areas of necrosis in the liver and spleen, pericarditis, and a yellowish fluid covering the brain surface can be commonly found (*Figure 2.4*) (Vendrell *et al.*, 2006; 2008).

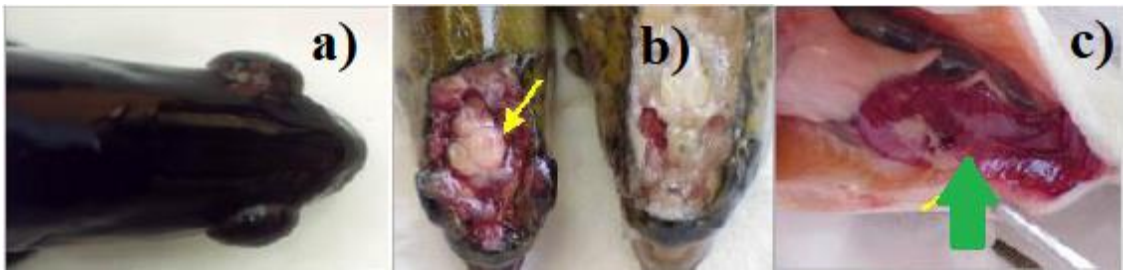

Figure 2.4. Rainbow trout presenting bilateral exophthalmia (a), yellow arrow pointing towards encephalitis (b), and green arrow pointing towards enteritis (c), caused by lactococcosis (*L. garvieae*). *Adapted from: Blanco et al. (2004).*

2.2.2.3. Yersiniosis (Enteric red-mouth disease)

The enteric red-mouth disease (ERM), caused by *Yersinia ruckeri*, is a disease that mainly affects salmonids of all ages and stages of growth, all over the world. This yersiniosis can cause heavy economic losses in affected farms, with a mortality rate reaching up to 70% of rainbow trout stocks. The disease was first reported in the United States of America (USA), in the decade of 1950, but since then, it has been reported worldwide. There are two different biotypes identified, *Yersinia ruckeri* serotype O1 biotype BT1, and the more recently discovered biotype BT2. The biotype BT1 is generally considered the most virulent one. The bacterial agent adheres and penetrates through mucosal surfaces, like the gut mucosa. As the name suggests, common lesions of the disease are subcutaneous haemorrhages in the mouth and throat of the rainbow trout. One of the most significant clinical diagnosis signals of enteric red-mouth disease is an

inflamed lower intestine. Other lesions can usually be observed, like exophthalmia, general congestion and presence of petechiae in the internal organs, haemorrhage and inflammation of the jaws and palate and even melanosis (*Figures 2.5 and 2.6*). The intestinal mucosa gets necrotic and edematous, filled in its lumen with yellow pus containing the bacteria and epithelial cells. Fish chronically infected with *Yersinia ruckeri* can spread the agent in the water through faeces (Blanco *et al*., 2004; Deshmukh *et al.*, 2012; Villumsen *et al.*, 2014; Gotesman *et al.*, 2018).

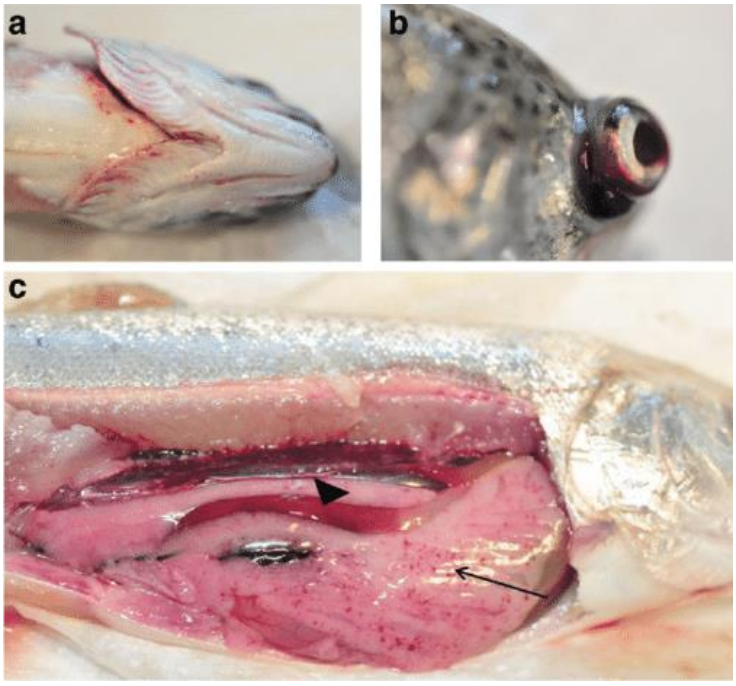

Figure 2.5. ERM lesions in rainbow trout: (a) petechiae and haemorrhages in the oral region; (b) exophthalmia; (c) the large arrow represents haemorrhages in the intestine and the thin arrow points to petechiae in the visceral tissue.
Source: Gotesman et al. (2018).

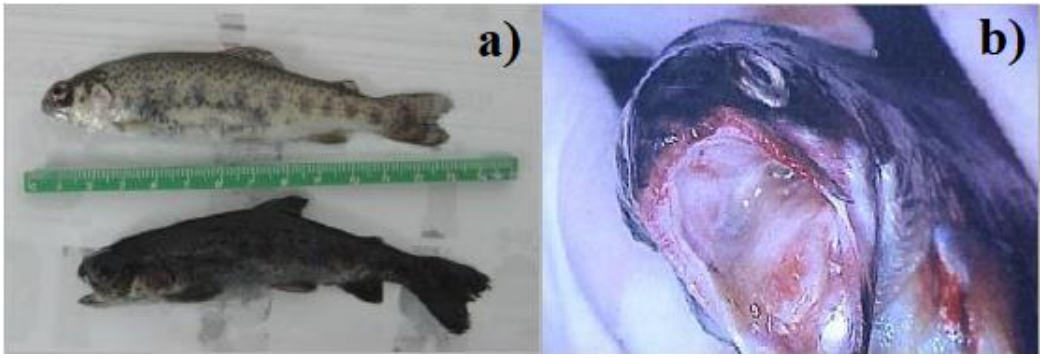

Figure 2.6. ERM lesions in rainbow trout: melanosis (a); bucal haemorrhages (b).
Adapted from: Blanco et al. (2004).

2.2.2.4. Furunculosis

In salmonids, namely the rainbow trout and the Atlantic salmon, the agent behind the classic furunculosis, is a non-motile, Gram-negative bacterium classified as *Aeromonas salmonicida* subsp. *salmonicida*. Formerly, the disease was very much associated with disease outbreaks in farmed salmonids. Nevertheless, nowadays it is known that the agent causes disease in many other fish families, in which the agent can manifest itself with different conditions, such as ulcerative dermatitis and other ulcerations. Salmonid's furunculosis is considered a very important disease, both in sanitary and economic terms. The disease is associated with some predisposing factors, such as the presence of external parasites, fish stock density or water temperature (especially above 16ºC). The presence of external parasites is an important issue, since the bacteria can enter the organism through skin lesions, disseminating itself through the bloodstream, spreading to multiple internal organs and establishing septicaemia. Depending both on environmental conditions and on the age and growth stage of the fish, furunculosis' severity can vary. Hyperacute forms of the disease mostly occur in fingerling fish, which sometimes last two to three days. In this form of presentation, frequently the only clinical findings might be melanosis and a slight exophthalmia. The acute form of the disease occurs especially in juvenile and adult fish. Lesions associated with it are usually melanosis, haemorrhages at the base of the fins and oral cavity, internal haemorrhages, enlarged spleen, subcapsular haemorrhages and focal necrosis on the liver. The affected fish might present an erratic swimming and anorexia. Sometimes during necropsy, it is possible to find the reproductive organs haemorrhaged and the intestine severely congested. The chronic and subacute forms of the disease are mostly found in older fish, characterized by a lethargic state, exophthalmia, bloody discharges from the nares and vent, and by multiple haemorrhages in the muscles and other tissues. The furuncles, that name the disease itself, are in fact not a consistent finding and associated with chronical presentations. The *Aeromonas salmonicida* subsp. *salmonicida* furuncles consist of tissue fluid exudate, necrotic tissue, and macrophages. In the most severe cases, there is a degeneration of the myofibrils, with muscle fibres fragmented, and muscle haemorrhages which can lead to a colliquative necrosis of the musculature involved. The furuncles can burst, leaving an open skin lesion that can lead to secondary infections (*Figure 2.7*) (Cipriano and Bullock, 2001; Blanco *et al.*, 2004; Austin and Austin, 2007).

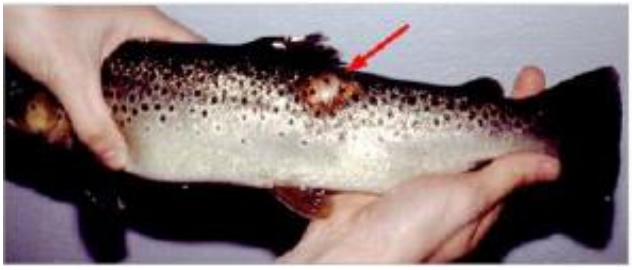

Figure 2.7. Presence of a furuncle in a rainbow trout (red arrow).
Source: Blanco *et al. (2004).*

2.2.2.5. Streptococcosis

In rainbow trout, streptococcosis is mainly caused by a Gram-positive bacterium *Streptococcus iniae*. Additionally, *Streptococcus agalactiae* has been also reported to cause disease in fish. It was first reported back in 1994 in Israel. Since then, the agent has been widely studied, and it has been demonstrated that it is widespread and that it affects multiple species, both fresh and saltwater. More recently, *Streptococcus iniae* has also been isolated from human blood, urine, and skin. It has been associated with some fatal course cases, being therefore considered an emerging pathogen with an increasing clinical significance in human medicine. In rainbow trout, the classic *Streptococcus iniae* infection is characterized mainly by panophthalmitis and meningitis, in a subacute or acute clinical picture. However, more recently a new strain has been described. Classified as serotype II, this new strain differs from the classical one (serotype I) in serological, phenotypical, and genetic criteria. *Streptococcus iniae* serotype II can enter the phagocytes and multiply inside of them, causing apoptotic processes. Common clinical signs and lesions associated with serotype II strains include lethargy, discoloration, loss of orientation, bilateral exophthalmia, corneal opacity, ocular haemorrhages, and both surface and internal haemorrhages (mostly in the spleen and fat around the intestine), which can eventually lead to death (Lahav, 2004; Austin and Austin, 2007).

2.2.2.6. Vibriosis

The taxonomic classification behind the main agent of fish vibriosis has suffered many changes and has been the centre of wide controversy for years. Referenced as early as 1893 (Canestrini, 1893), as the *red disease* of eels, the agent was classified back then as *Bacterium anguillarum.* Few years later, in 1909, Bergman (1909) proposed a new classification, as *Vibrio anguillarum*. In 1984, MacDonell and Colwell (1985) while

studying variations among the *5S rRNA* region of *Vibronaceae* bacteria proposed a revised classification for the agent, as *Listonella anguillarum*. More recently, in 2011, it was once again reaccepted the former classification as *Vibrio anguillarum* (Dikow, 2011; Thompson *et al.,* 2011). Thus, at the present time, the most consensual classification, as the controversy still exists, appears to be *Vibrio (Listonella) anguillarum*. This is a Gram-negative, comma-shaped rod bacterium, that it is pathogenic to at least 90 different aquatic organisms. Other *Vibrio* spp., such as *Vibrio ordalii* (formerly known as *Vibrio anguillarum* biotype 2), are also associated with similar clinical vibriosis outbreaks in fish. The agent's virulence has gained increased prevalence mostly due to the exposure of farmed fish to stress factors. These stress factors include water quality and temperature, especially higher temperatures (above 10ºC), pollution, stock density, and the presence of other microorganisms. Stress activated virulent strains appear to be the ones causing the most damage, since the bacteria can be found among the normal gut microflora of healthy fish. Some reports have pointed to mortality rates reaching 100% in vibriosis outbreaks. Vibriosis is characterized by a haemorrhagic septicaemic disease, affecting both fresh and brackish water species. External clinical signs include weight loss, lethargy, petechiae in ventral and lateral areas, haemorrhages near the base of pectoral and pelvic fins, melanosis, and skin ulcers. Ophthalmologic problems can also appear, initially with opacity, followed by periorbital oedema, ulceration and exophthalmia. The pathogen can be found in high concentrations in the blood and haemopoietic tissues. However, in hyperacute or acute outbreaks the disease can spread so fast that most of the infected fish die without showing clinical signs. Once the infection is present in one pond or tank, it spreads horizontally (Frans *et al.,* 2011; Bruno *et al.,* 2013; Hickey and Lee, 2017).

2.2.2.7. Bacterial kidney disease (BKD)

The BKD is caused by a small Gram-positive bacillus named *Renibacterium salmoninarum*. It is considered one of the most prevalent bacterial diseases in reared salmonids, including the rainbow trout. BKD is a fastidious, slowly progressive systemic infection, in part due to the inherent and natural slow growth of the bacterial agent. Clinical signs might take months to appear after infection. BKD is characterized by a chronic, systemic, granulomatous infection. The agent produces grey-white necrotic abscesses in the kidney (initially beneath the kidney's capsule, on the ventral side) which,

in advanced and severe cases, can affect and involve the entire kidney. Most of the epizootics occur during periods where the water temperature declines (autumn and winter). Nevertheless, the mortality has been registered higher during periods of warmer water temperatures, which indicates that BKD is a disease that occurs over a wide range of water temperatures. External signs of the pathology are usually only observed in its final stages. Some of these signs include exophthalmia, abdominal distension, superficial blebs or blisters, abscesses, and haemorrhages all over the body surface. BKD can be transmitted either horizontally, through contact with infected fish or contaminated water, and vertically, from infected broodstock eggs (Fryer and Sanders, 1981; Bruno *et al.*, 2013).

2.2.2.8. Pseudokidney disease

Carnobacterium maltaromaticum (formerly known as *Carnobacterium piscicola*), is the Gram-positive aetiological agent that causes the disease often referred to as pseudokidney disease. This bacterium has been isolated from apparently healthy reared and wild fish (*Oncorhynchus* spp.), with unexpected high prevalence in feral stocks. Therefore suggesting that the agent is part of the normal microflora of the gastrointestinal tract. Stress factors such as handling, spawning and post-spawning periods, among others, are considered as triggers for the development of a disease that tends to have a chronic course. Clinical signs are not considered consistent, but include septicaemia, visceral congestion, opacity and thickening of the swim-bladder, abdominal distension, bilateral exophthalmia, periocular haemorrhages, and external ulcerations. Additionally, internal signs consistent with nephrocalcinosis, such as renal swelling, dilation and presence of white granular substance in the ureters, mineralized tubules, presence of multifocal clusters of white nodules, and renal tubular degeneration, may well be observed (Loch *et al.*, 2011; Bruno *et al.*, 2013).

2.2.2.9. Rainbow trout fry syndrome (RTFS)

The rainbow trout fry syndrome (RTFS), also referred to as bacterial coldwater disease (CWD) in the USA or even peduncle disease, is an important acute septicaemic infection in salmonids. Its causative agent is a Gram-negative bacterium, *Flavobacterium psychrophilum*. It is an infection commonly associated with low water temperatures (4-

10ºC), hence, the North American designation of CWD. The severity of the disease is related with the age of the fish. Usually, more severe infections are related to younger fish stocks. Among alevins, the mortality rate can range from 30 to 50%. Clinical signs common among fry, fingerlings and juveniles are lethargy, reduced appetite, and dark colouration. The causative agent has a certain affinity for skin and muscle tissues, which can originate yellowish lesions on the caudal peduncle region. This lesion can progress deeper into the muscle, eventually becoming necrotic. One special concern is the possibility of spinal deformities, such as spinal compressions, in surviving individuals. This poses a threat in obtaining the correct market-sized fish or even undesired structural shapes for industrial processes, which can lead to hypothetical economic losses (Wood and Yasutake, 1956; LaFrentz and Cain, 2004;).

2.3. Disease control measures

2.3.1. Antibiotics

In the aquaculture industry the use of antibiotics, either as therapeutic or prophylactic agents, has been widespread. In veterinary medicine the use of these drugs is in many countries unregulated and unrestricted, as well as very variable among them. For instance, it is estimated that in Norway the antibiotic consumption is 1 gram (g) per tonne, whereas in Vietnam it is 700 g per tonne. In the rainbow trout rearing process, as well as with other species, handling and manipulation can act as stress factors. Consequently, the effectiveness of the fish's immune system can be compromised, which can eventually lead to colonization and infection by opportunistic agents. Likewise, sanitary and hygienic deterioration due to inappropriate fish raising methods, high stock densities, crowding of farming sites, lack of sanitary barriers, among others, have widely contributed to the emergence of infectious outbreaks in farming facilities. Therefore, the use of antibiotics as a prophylactic measure has been a common policy among farmers. Typically, antibiotics are given to fish through food, but occasionally they can be given through injections or even through bath immersions. There are several issues associated with the excessive use of antibiotics in aquaculture. Firstly, the increasing occurrence and transfer of antibiotic resistance (sometimes multiple antibiotic resistances) in pathogenic bacteria of the fish's microbiota, which sometimes can harbour new and uncharacterized resistance determinants. Ultimately, this increasing occurrence and transfer of antibiotic

resistances could not only lead to an increase of fish bacterial diseases, but also undermine the effectiveness of current therapeutic antibiotic usage whenever needed. Secondly, the increasing possibility of exchanging these antibiotic resistance determinants with both bacteria of terrestrial animals and humans, which some of them might be pathogenic. This exchange can occur horizontally, through mechanisms such as conjugation (as plasmid mediated transfers, which have been widely reported in fish pathogens) and conjugative transposition. Transfer through transduction can also pose an important role, due to the high concentration of viruses in the aquatic environment, especially in seawater and marine sediment. Furthermore, the presence of antibiotic residues in commercialized fish and shellfish can lead to the undetected consumption of antibiotics. Undesired effects can range from toxicological and allergic reactions to intestinal dysbiosis, not only on consumers but also on fish industry workers (through contact with skin, gastrointestinal and bronchial tracts). Lastly, the unconsumed food and fish's faeces containing antibiotics, which reach the sediment of the ponds, exert selective pressure on the habitat's microbiota. In mariculture and coastal farming sites, the ocean currents can wash the sediments to the environment, eventually entering in new food chains. Additionally, there is also the problem associated with freshwater farms, that can release their effluents on river streams, spreading it to the surrounding downstream environment and even to surrounding farms (Dixon, 1994; Guardabassi *et al.*, 2000; Schmidt *et al.*, 2000; Cabello, 2006; EFSA, 2008a; 2008b; Ringø *et al.*, 2014).

During the decades of 1970 and 1980, antibiotics such as the oxolinic acid, oxytetracycline, furazolidone, potentiated sulphonamides (sulphadiazine and trimethoprim), and amoxicillin were amongst the most used in aquaculture practice, sometimes used in subtherapeutic doses (Hatha *et al.*, 2005; Ringø *et al.*, 2014;). More recently, antibiotics such as streptomycin, erythromycin and chloramphenicol are used as aquaculture therapeutic agents, while oxytetracycline and penicillin are used as prophylactic agents. Progressively, this led to selective pressure of antibiotic resistance in bacteria, as stated before. Ultimately, the decreased efficiency of such therapeutic or prophylactic protocols are not new to the sector and have been reported for years. Multiple antibiotic classes and groups, with different mechanisms of action, have been associated with resistance determinants, including: antibiotics that target the protein synthesis (oxytetracycline, erythromycin, chloramphenicol and streptomycin), antibiotics that inhibit cell wall synthesis (ampicillin and penicillin), antibiotics that inhibit nucleic acid synthesis (quinolones) and the ones that inhibit folic acid synthesis (sulphonamides) (Teo

et al., 2000; Hatha *et al.*, 2005; Akinbowale *et al.*, 2007; Ringø *et al.*, 2014). Regarding this, some of the bacteria previously mentioned as commonly involved in rainbow trout infectious outbreaks have been associated with antibiotic resistances, such as *Aeromonas salmonicida*, *Yersinia ruckeri*, *Vibrio (Listonella) anguillarum*, and *Flavobacterium psychrophilum* (de Paola *et al.*, 1995). In (Balta *et al.*, 2010), for instance, an abundant presence (around 51% of the strains screened) of tetracycline resistance determinants, *tet*(A) and/or *tet*(B) genes, was reported in *Yersinia ruckeri* isolated from rainbow trout commercial farms, in Turkey. Also, (Kawanishi *et al.*, 2005) confirmed the presence of resistance genes *ermB* and *tet*(S), in *L. garvieae* obtained from cultured fish (genus *Seriola*) suffering from lactococcosis in Japan. Those genes conferred erythromycin, lincomycin and oxytetracycline resistance to those strains. Other fish pathogens, not particularly related to rainbow trout farming, have been too for years associated with antibiotic resistance determinants (de Paola *et al.*, 1995). For instance, (Ishida *et al.*, 2010) described the presence of tetracycline resistance genes *tet*(A), *tet*(C) and *tet*(E) in *Aeromonas hydrophila*, as well as genes conferring resistance to β-lactam antibiotics (such as penicillins and first-generation cephalosporins) bla_{TEM}, plasmid-mediated quinolone resistance gene (*qnrS*), chloramphenicol resistance gene (*catB3*) and trimethoprim resistance genes (*dfrA7*), in *Aeromonas hydrophila* strains isolated from brackish water fish farms in northern Egypt.

Moreover, the risk of transferring antibiotic resistance determinants from fish pathogens to terrestrial animals and human pathogens is real. It represents a key issue involving an excessive and erroneous usage of antibiotics in aquaculture, and, in fact, in any other form of husbandry (Cabello, 2006). In aquaculture, tetracyclines have been frequently used as a treatment for furunculosis (*Aeromonas* spp.), which led inevitably to the emergence of tetracycline resistances. Considering that the presence of a tetracycline resistance determinant transposon (*Tn1721*) has been formerly demonstrated, previously disseminated among the fish pathogen *Aeromonas salmonicida*, in human pathogens such as *Aeromonas hydrophila*, *Aeromonas caviae* and *E. coli*, isolated from fish farms and human hospitals (Rhodes *et al.*, 2000). Furthermore, molecular and epidemiological evidence suggests that some resistant determinants of multi-resistant *Salmonella enterica* serotype typhimurium DT104 (MR-DT104), considered an emergent veterinary and human pathogen, can have their origin traced back to aquaculture farms in Asia. Florfenicol has been regularly used in aquaculture in Asia since the decade of 1980. The MR-DT104 florfenicol resistance determinant, *floR*, was detected originally in a fish

pathogen called *Photobacterium damselae* (previously known as *Vibrio damsela*). Likewise, the MR-DT104 tetracycline resistant determinant, a class G resistance gene, was also initially detected in other fish pathogen, *Vibrio (Listonella) anguillarum*. This evidence suggests that some of the resistance determinants of MR-DT104 may have emerged from aquaculture pathogens and been horizontally transmitted to it (Angulo, 2000; Angulo and Griffin, 2000; Cabello, 2006). Several other pieces of evidence between aquaculture and animal or human pathogens antibiotic resistance determinants exchanges exist, which together support the theory that aquaculture and human compartments of the environment behave as an interactive compartment, rather than isolated ones (Rhodes *et al.,* 2000).

The urge to combat excessive and erroneous antibiotic usage in aquaculture has led industrialized and developed countries, such as those forming the EU, USA, Canada, or Japan to implement strict regulations about it. In addition, some governments around the world have also imposed Maximum Residue Levels (MRL) for aquaculture products. The regulations imposed by those countries include measures such as an increased control over veterinary prescriptions of therapeutic antibiotics, an almost total elimination of antibiotic prophylaxis and proscription of the use of antibiotics relevant in human infectious diseases (Cabello, 2006; Defoirdt *et al.,* 2011). Hence, the EU's authorized list of antibiotics in aquaculture practice has been narrowed down to seven drugs: amoxicillin, florfenicol, flumequine, oxolinic acid, oxytetracycline, sarafloxacin and sulfadiazine-trimethoprim (Furones and Rodgers, 2009). Nevertheless, countries with strong aquaculture sectors, such as China and Chile, continue to use relevant antibiotic groups without restrictions, like the quinolones (Cabello, 2006).

2.3.2. Vaccination

Vaccination has widely contributed to the reduction of losses related with infectious outbreaks in aquaculture. Additionally, it also represents a major factor that contributed to the reduction of antimicrobial compounds in aquaculture (EFSA, 2008b). The first fish vaccine being commercially available dates from 1976, developed to provide protection against *Yersinia ruckeri*, responsible for ERM. Vaccination has a major role in the success of large-scale commercialization of farmed fish. Initially applied and developed for salmonids (Atlantic salmon and rainbow trout), nowadays, vaccines are available for at least 17 species of fish, offering protection against more than 22 different bacterial agents

and 6 viral agents, in more than 40 countries. Nevertheless, in China, the world's biggest aquaculture producer, vaccination is not yet a common practice. Among the farmed species with current practice and availability of vaccination are the Atlantic salmon, rainbow trout, gilt-head seabream, Nile tilapia, turbot, seabass, Japanese amberjack (*Seriola quinqueradiata*), channel catfish (*Ictalurus punctatus*), or the Atlantic cod (*Gadus morhua*). Various bacterial diseases that affect the rainbow trout farming process currently have commercial vaccines available, such as vibriosis [*Vibrio (Listonella) anguillarum*], furunculosis (*Aeromonas salmonicida* subsp. *salmonicida*), yersiniosis (*Yersinia ruckeri*), streptococcosis (*Streptococcus iniae*), and lactococcosis (*L. garvieae*). Furthermore, experimental vaccines are being used in salmonids against BKD (*Renibacterium salmoninarum*) (Brudeseth *et al.,* 2013; Ringø *et al.,* 2014;).

Vaccination can usually be performed by three different methods. Firstly, it can be administrated orally, through feed. Additionally, the vaccine can also be diluted in a suspension, therefore administrated through immersion. The last method is given through injection, either intraperitoneal or intramuscular. The injection methods apparently provide longer and more efficient protections. Nevertheless, it is also the most stressful method and the one requiring most handling. Therefore, fries, being too small to be handled and injected, are usually vaccinated either through immersion or orally (Brudeseth *et al.,* 2013).

Although vaccination has been widely beneficial and advantageous to aquaculture, there are some key points concerning animal welfare, mostly regarding vaccination through injection methods, that could present disadvantages. The handling process associated with vaccination poses itself a stressful moment for the fish. Additionally, some evidence points out that unvaccinated Atlantic salmon gain up to one kilogram more than vaccinated ones, under the same rearing conditions, during the same period. Most of the times, the vaccine's adjuvant enhances the immune response to most parenteral fish vaccines. This adjuvant causes a chronic inflammatory response at the site, attracting macrophages, lymphocytes and enhancing blood supply, which together ensure the maximal uptake of the antigens by the host, enhancing the efficacy of the vaccine too. This chronic inflammatory response leads to melanisation of the local tissue. Melanisation can be a quality issue, which can diminish its market value for the consumer. The injection site also suffers fibrosis, contracting as a collagenous scarring develops. Both fibrosis and melanosis are assessed on a scale known as the Spielberg scale. Peritoneal adhesions probably represent the major welfare issue relating to injectable

vaccines. Recently, some progress has been made in developing vaccines with the use of less aggressive oils as adjuvants (EFSA, 2008a).

2.3.3. Immunostimulants

Immunostimulants are compounds that enhance non-specific defence mechanisms of fish, increasing their immunocompetency and resistance to infectious diseases. The fish's defence system is similar to that of mammals, with both cellular (phagocytic cells similar to macrophages, neutrophils, lymphocytes T and B and natural killer cells) and humoral (complement, lysozyme, C-reactive protein, natural haemolysin, and some cytokines have been reported) defence systems. Immunostimulants can be divided into five groups according to their nature, i) synthetical chemical agents, such as levamisole and FK-565; ii) bacterial derivatives, such as poly- and lipopolysaccharides, peptidoglycans, muramyl dipeptides and Freund's complete adjuvant; iii) animal, plant and algae extracts; iv) diet components, such as vitamin C and E; v) hormones, cytokines and lactoferrin. The routes of administration are similar to those of vaccination. Therefore, the main routes are injection (although considered a labour-intensive, time-consuming, and impractical method on smaller fish), oral administration, immersion, and even administered as vaccine adjuvants (Sakai, 1999). Immunostimulants such as β-glucans (a yeast derived product) used as feed additive for several years, alginate (a polysaccharide compound), and Ergosan [a brown algae (*Laminaria digitata*) based product, possessing high levels of alginate and polysaccharides], are currently the most promising ones in aquaculture (Peddie *et al.*, 2002; Ringø *et al.*, 2014;). For instance, in (Peddie *et al.*, 2002) it was demonstrated that a single dose peritoneal administration of 1 mg of Ergosan in rainbow trout increased the proportion of neutrophils, degree of phagocytosis, respiratory burst activity, expression of interleukin-1β and interleukin-8, and one of the two known isoforms of trout tumour necrosis factor-α, in peritoneal leucocytes one day after the peritoneal injection.

2.3.4. Bacteriophages (Phage therapy)

According to (Romero *et al.*, 2012), bacteriophages are viruses that can infect, multiply in and kill susceptible bacteria. Viruses are ubiquitous and abundant, especially in seawater. Since 1915, the year viruses' phage ability was discovered, their therapeutic

properties have been studied both in human and veterinary medicine. Nevertheless, due to the discovery and introduction of cheap, broad spectrum antibiotics they were progressively abandoned. More recently, as a result of the increasing interest in finding alternatives to the recurring use of antibiotics, phage therapy has regained some attention. Advantages associated with phage therapy over other therapeutic agents, namely antibiotics, include i) narrow host range of phages, indicating that they probably do not harm the normal intestinal microflora of the animal; and ii) phages can self-replicate inside susceptible bacteria, which discards the need for multiple administrations (Nakai and Park, 2002). The first experiment involving phage therapy applied to aquaculture and fish pathology is attributed to (Nakai *et al.,* 1999), which demonstrated the ability of phages to prevent lactococcosis (caused by *L. garvieae*) in Japanese amberjack. Since then, multiple studies have been carried out regarding phage therapy and aquaculture. For instance, more recently (Imbeault *et al.,* 2006) reported the success of the bacteriophage HER110 on preventing *Aeromonas salmonicida* (furunculosis) infection and reducing its mortality rate in brook trout (*Salvelinus fontinalis*). Moreover, a phage can be active against multiple sensible targets as demonstrated in (Vinod *et al.,* 2006), where 50 *Vibrio harveyi* isolates were sensitive to a single phage. However, constraints such as the possible transfer of virulence factor through phages and the rapid development of resistance to phage attachment, currently pose some limitations to their widespread use as therapeutic agents (Defoirdt *et al.,* 2011).

2.3.5. Prebiotics

The prebiotic concept was established by (Gibson and Roberfroid, 1995), as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria (…) and thus improves host health". The prebiotics act as substrates or energy sources for beneficial commensal bacterial, which due to processes such as fermentation exert an important role enhancing the host health (Gibson and Roberfroid, 1995; Song *et al.,* 2014). Later, Kocher (2004) suggested the existence of a distinct type of prebiotics, the immunosaccharides. Immunosaccharides differ from the classical category of prebiotics because they stimulate the fish innate immune system directly, instead of via fermented products. Substances commonly regarded as prebiotics include oligosaccharides such as fructooligosaccharides (FOS), mannan-oligosaccharides (MOS), galactooligosaccharides (GOS), arabinoxylan-

oligosaccharides (AXOS), and polysaccharides such as inulin (its hydrolysis by specific bacteria produces FOS) and β-glucans (glucose polymer linked through β-glycosidic bonds). Several experiments concerning prebiotic application on aquaculture have been conducted, involving fish families such as *Salmonidae*, *Sciaenidae*, *Cyprinidae*, among others (Song *et al.*, 2014).

2.3.6. Probiotics

Probiotics definition has been used in several ways throughout the years and has been continually changing since its first references, as new findings emerge. The term probiotic was initially used by Lilly and Stillwell (Lilly and Stillwell, 1965) to describe species of protozoan that produced substances that stimulated other species. Later, (Parker, 1974) used the term probiotic to refer "organisms and substances which contribute to intestinal microbial balance". A few years later, Fuller (Fuller, 1989) revised the definition of probiotic into "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance", also stating that a probiotic should be harmless to its host. A probiotic should be, therefore, neither invasive nor pathogenic to a host. More recently, a joint FAO/WHO report recommended the definition of probiotic as: "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2002). The use of probiotics is considered a serious and important alternative strategy to antibiotic prophylaxis and therapeutic, and their use in human and veterinary medicine, including aquaculture, has been experimented and well documented over the last decades (Balcázar *et al.*, 2006; Pérez-Sánchez *et al.*, 2014). The use of probiotics as prophylactic agents should be faced as a "risk insurance", with no remarkable effects under optimal conditions of rearing and in the absence of pathogens (especially in the absence of opportunistic ones), but valuable and helpful during infectious outbreaks (Verschuere *et al.*, 2000a). During the next topic section (number 4), probiotics, their diversified mechanisms of action and their application in aquaculture will be further developed.

The inclusion of prebiotics in aquaculture feeding practices can be done singly or jointly with probiotics. Additionally, prebiotics and probiotics combinations can work synergistically, as symbiotics, increasing and enhancing the survival and gut implantation of probiotic microorganisms, improving the host health and welfare (Gibson and Roberfroid, 1995; Cerezuela *et al.*, 2011; Huynh *et al.*, 2017).

2.4. Probiotics in aquaculture

2.4.1. Definition applied to aquaculture

The definition stipulated by Fuller, when applied to aquaculture has required some considerations. Aquatic animals, such as farmed fish, live in an aquatic environment surrounded by water. This means that both hosts and microorganisms constantly share the same environment. In fact, the water that hosts the animal is the same water that gives support to microorganisms, that might be pathogenic, in some cases reaching high densities. Those microorganisms contact not only the gastrointestinal tract but also with the skin and gills. Therefore, in aquaculture, the environment stands as more influential to the host and its health status than in land animals or humans. Aquatic organisms usually after spawning do not have further contact with their parents. Besides not having a fully developed gastrointestinal tract, they also do not have a fully stable microbial community in the gut, skin, or gills. Hence, the quality of the rearing water and the properties of the microbial community in it are crucial to the young fish' health. In aquatic environments, the interactions between microbiota and hosts are not limited to the gastrointestinal tract but are also very strong in their skin and gills. Considering this, the definition of probiotic has been adapted regarding the aquaculture sector. Hence, probiotics are live microbial adjuncts that have beneficial effects on the host by: i) "modifying the host-associated or ambient microbial community"; ii) "ensuring improved use of feed or enhancing its nutritional value"; iii) "enhancing the host response towards disease"; and iv) "improving the quality of its ambient environment" (Verschuere *et al.*, 2000a).

2.4.2. Safety assessment for selection of a probiotic

The selection of a microorganism or mixture of microorganisms is a complex and multidisciplinary process. It requires empirical research, trials, as well as economic studies to evaluate its practical commercial application (Verschuere *et al.*, 2000a). Currently there are no specific guidelines for the use of probiotics in aquaculture. However, a joint working group of FAO/World Health Organization (WHO) has, for the first time, published general recommendations for the assessment of the use of probiotics in food, addressing a needed thematic (FAO/WHO, 2002). According to the FAO/WHO document, the following steps should be taken when assessing the use of a probiotic in

food: i) microorganism and strain identification by phenotypic and genotypic methods; ii) functional characterization of the microorganism (*in vitro* and *in vivo* tests); iii) safety assessment (*in vitro* and *in vivo* tests), which should include determination of antibiotic resistance determinants, virulence factors, assessment of certain metabolic activities (like bile salt deconjugation), assessment of side effects, haemolytic potential, and epidemiological surveillance (post market step); iv) effectiveness trials, results confirmation (preferably with an additional independent one), and finally v) labelling, presentation and commercialization. Moreover, with Regulation (EC) No. 178/2002 the European Parliament established the standards for a common policy over food safety for the EU and created the European Food Safety Authority (EFSA). Since its establishment, EFSA has progressively published several documents about the Qualified Presumption of Safety (QPS) status, addressing safety assessment of microorganisms used in food and feed, taxonomical identifications, and assessment of bacterial susceptibility to antimicrobial agents of importance in human and veterinary medicine (EFSA, 2005a; 2005b; 2007; 2012; 2020). Although the guidelines established by these world institutions are not specifically addressed to aquaculture, they have established a precedent and a standard for the so needed research in the area (Cruz *et al.*, 2012). Consequently, nowadays in the EU, the authorization, commercialization, marketing and use of a probiotic as a feed supplement is regulated under Regulation (EC) No. 1831/2003 and Regulation (EC) No. 767/2009 (which substituted the former Council Directive 70/524/EEC), that follow the guidelines issued by the Scientific Committee on Animal Nutrition (SCAN) of EFSA (Irianto and Austin, 2002a; von Wright, 2005; Balcázar *et al.*, 2006).

2.4.3. Functional, technological properties and efficacy

Potential probiotic microorganisms with beneficial health effects on the host must meet some criteria, such as functional and efficacy properties like: i) a probiotic candidate must be able to survive passage through the gastrointestinal tract, resisting or expressing, for instance, high tolerance to acid pH, bile, and proteases; ii) the probiotic candidate should be able to adhere to the intestinal mucosa in order to colonize, multiply and persist inside the gastrointestinal tract; considering the specificities of an aquatic environment, a potential probiotic, in aquaculture, should also demonstrate the capacity to colonize surfaces, such as the skin; iii) a probiotic candidate should demonstrate efficient

antimicrobial activity or antagonistic properties (*e.g.,* production of antagonistic compounds) when challenged against a potential pathogen (*in vitro* and *in vivo* studies should both be carried out); iv) it is desirable to observe an increase in growth rate, as well as a decrease in mortality on target host, after being challenged with a pathogen; v) enhancing the fish immune system, as well as both cellular and humoral immune response has been also proven and it is considered a desirable characteristic when assessing the efficacy of a potential probiotic; vi) considering that the final step for a potential probiotic would be commercialization, a probiotic candidate should be also viable under storage conditions and should resist industrial technological processes, such as lyophilization. During the selection process of a probiotic, *in vitro* screening experiments are vital to optimize the conditions (feeding doses, viability, among others) to perform *in vivo* challenges. It is noteworthy that before commercialization, it is also highly recommendable to realize a cost-benefit analysis, to ensure market and economic viability (Verschuere *et al.,* 2000a; Nikoskelainen *et al.,* 2001a; Balcázar *et al.,* 2006; 2008; Kim and Austin, 2006; Merrifield *et al.,* 2010a; Pérez-Sánchez *et al.*, 2014).

2.4.4. Mechanisms of action of probiotics

Although over the past few decades, several studies have been made concerning probiotics and their effects on the host health status, there are multiple obstacles limiting a full understanding on their mechanisms of action. Therefore, only partial theories and explanations are currently available. In spite of the increasing interest on probiotics, explanations for their mechanisms of action have been only circumstantial throughout the decades. Nevertheless, the knowledge gathered with the use of probiotics in humans and terrestrial animals has been applied in aquaculture, especially when regarding the use of LAB as probiotic agents. Despite the uncertainty, the mechanisms by which probiotics exercise their effects in aquaculture can be currently organized in the following groups: i) bacterial antagonism by competitive exclusion; ii) improvement of the host nutrition and enzymatic contribution to digestion; iii) enhancement of the fish immune response; iv) improvement of water quality; and lastly v) tolerance to stress factors (Verschuere *et al.,* 2000a; Balcázar *et al.,* 2006; Carnevali *et al.,* 2006; Merrifield *et al.,* 2010a; Nayak, 2010, Gómez-Sala *et al.,* 2019).

2.4.4.1. Competitive exclusion

2.4.4.1.1. Production of antimicrobial compounds

Bacterial antagonism is a constant interaction in nature, that shapes the equilibrium between microbial communities. This is of special importance when it comes to the equilibrium between competing beneficial microorganisms and potentially pathogenic ones, in a host. The ability of bacteria found in aquatic environments to inhibit other bacteria growth is not an uncommon feature. Consequently, the phenomenon has been known for more than a century, being first described in as early as in the 19th century. A few decades later, (Rosenfeld and Zobell, 1947) described either a bactericidal or bacteriostatic activity of seawater on nonmarine bacteria, in culture, reporting the existence of antibiotic-producing marine microorganisms in it. Nevertheless, there are other ways probiotics can exert antagonistic or inhibitive properties rather than antibiotic production. The effect can also be achieved by the production of other substances such as antimicrobial peptides (*e.g.* bacteriocins), lysozymes, proteases, hydrogen peroxide, siderophores, organic acids, iron chelating compounds, ammonia, and acetyl. For instance, it is vastly documented that multiple LAB, like those of the following genera *Carnobacterium, Lactococcus, Lactobacillus, Enterococcus* or *Leuconostoc*, have the ability to inhibit some pathogenic bacteria growth via bacteriocin-production (Verschuere *et al.*, 2000a; Cintas *et al.*, 2001; Balcázar *et al.*, 2006; Pérez-Sánchez *et al.*, 2014).

2.4.4.1.2. Competition for adhesion sites

The ability to adhere to intestinal mucus and tissue surfaces is essential for a proper colonization of the gastrointestinal tract. Bacterial adhesion is regarded as one of the initial steps of a pathogenic infection, therefore, the ability of a candidate probiotic to adhere to binding sites, mucus or other tissue surfaces (such as the skin or gills) is considered a desirable characteristic and criterion when assessing its early-stage prevention potential. The adhesion process can be either non-specific or specific. Non-specific adhesion relies on physicochemical factors, while specific adhesion implies the presence of adhesin molecules on the surface of the adherent bacteria and the existence of receptor molecules on the target tissue surface (Verschuere *et al.*, 2000a; Balcázar *et al.*, 2006; Pérez-Sánchez *et al.*, 2014). Considering this, several experiments have been

conducted demonstrating *in vitro* ability of probiotic strains to inhibit the adhesion of pathogens to fish intestinal mucus. For instance, (Balcázar *et al.*, 2008) has demonstrated the ability of three LAB (*Lactococcus lactis* CLFP 101, *Lactobacillus plantarum* CLFP 238 and *Lactobacillus fermentum* CFLP 242) to compete for binding sites and inhibit the adhesion of fish pathogens (*Aeromonas hydrophila*, *Aeromonas salmonicida*, *Yersinia ruckeri* and *Vibrio (Listonella) anguillarum*) to intestinal mucus of rainbow trout.

2.4.4.1.3. Competition for nutrients

In aquaculture, the microbiome is dominated by heterotrophs that compete among each other for nutrients and energy sources, such as organic substrates. Theoretically, it is likely that competition for nutrients and energy sources operates a major role in the interactions between coexisting aquatic microorganisms. Nevertheless, few studies have demonstrated this form of competitive exclusion as it is proposed (Verschuere *et al.*, 2000a). However, (Rico-Mora *et al.*, 1998) demonstrated that a bacterial strain (SK-05, likely *Aeromonas* sp.) presenting active growth in organic-poor substrates, and with no bacteriostatic or antibiotic activity towards *Vibrio alginolyticus*, prevented the establishment of the latter one in diatom culture. Hence, it was suggested that the strain outcompeted *Vibrio alginolyticus* by competitive exclusion, utilizing the exudates of the diatoms. Likewise, (Verschuere *et al.*, 2000b) suggested that selected bacterial strains (classified as LVS2 and LVS8), while not producing extracellular compounds such as antibiotics, exerted inhibitory effect against a virulent pathogen of *Artemia* spp., identified as *Vibrio proteolyticus* CW8T2, by competing for chemicals or available energy.

2.4.4.2. Improvement of the host nutrition

Another possible mechanism of action suggested is that probiotics can have a direct effect on the host growth rate by improving its nutrition. This nutritional improvement can occur either through providing nutrients, such as proteins, short chain fatty acids, vitamins (such as vitamin B_{12}, C and K), among others, or either through the enhancement of enzymic activity, that ultimately leads to a more efficient digestion and to better weight gains (Vine *et al.*, 2006; Pérez-Sánchez *et al.*, 2014). Regarding this mechanism of action, some *in vivo* experiments attesting the important of probiotics for nutritional parameters

of the host have been carried out. For instance, (Merrifield *et al.,* 2010b) demonstrated that a 10 week use of probiotic strains (a mixture that included *Bacillus subtilis* and *Bacillus licheniformes*) as feed supplement for rainbow trout, had a significant positive impact on nutritional parameters, such as feed conversion ratio, specific growth rate and protein efficiency ratio. Furthermore, in (Tovar-Ramírez *et al.,* 2004) groups of seabass fed with live yeast (*Debaryomyces hansenii* CBS 8339) reported higher levels of activity and concentration of mRNA trypsin and lipase, and higher levels of activity of intestinal enzymes, such as alkaline phosphatase, aminopeptidase N and maltase. The positive effects on larval performance registered, which included an increase in larvae growth, could be due to polyamines secreted by the yeast, which promote intestinal maturation, and increase nutrients absorption by the enterocytes.

2.4.4.3. Enhancement of the immune response

The earlier experiments conducting probiotic effects on fish mainly aimed for nutritional parameters, such as growth performance, and disease protective abilities. Nevertheless, more recently, some attention has been directed towards the immunomodulating effects of probiotics on fish immune system and response, both systemic and local. Thus, the effects of probiotics in modulating the immune system are now regarded as one of the most common benefits of probiotics in aquaculture. These effects can range from cytokine production, phagocytic activity, respiratory burst activity, peroxidase activity, lysozyme activity, complement activity, to even immunoglobulin production. The immunomodulation effects on fish obtained using probiotic bacteria have been assessed in several immunological studies (Harikrishnan *et al.*, 2010; Nayak, 2010; Pérez-Sánchez *et al.,* 2014). For example, (Sakai *et al.,* 1995) described an increase on phagocytic activity of leucocytes, in rainbow trout fed with *Clostridium butyricum* bacterin for three consecutive days, showing an enhanced resistance towards *Vibrio (Listonella) anguillarum* after inoculation. Moreover, (Kim and Austin, 2006) reported that the use, as feed supplement, of two bacterial strains (*Carnobacterium maltaromaticum* and *Carnobacterium divergens*) enhanced both cellular and humoral responses of rainbow trout. An increased phagocytic activity of the head kidney macrophages, an increased respiratory burst, as well as an increased serum and gut lysozyme activity were registered.

2.4.4.4. Improvement of water quality

Probiotics when added to the rearing water can act both as biocontrol agents and as bioremediation agents, improving the water quality of the environment (Taoka *et al.*, 2010). The ability to improve water quality has been mainly linked to the use of Gram-positive bacteria, such as *Bacillus* spp. They are regarded as better organic matter converters than Gram-negative bacteria, which would rather convert a large percentage of it into bacterial biomass or slime. Therefore, it is expectable that by maintaining interesting levels of these Gram-positive bacteria in the ponds, the accumulation of dissolved and particulate organic carbon can be minimized during the culture cycles, boosting the production of CO_2. Additionally, nitrifying cultures can also be added to the rearing ponds, especially whenever unusual toxic increases of ammonia and nitrites concentrations occur. Nitrifying cultures can help in the oxidation of ammonia into nitrite, and subsequently into nitrate (Verschuere *et al.*, 2000a; Balcázar *et al.*, 2006). (Dalmin *et al.*, 2001) observed that the application of *Bacillus* spp. into extensive shrimp (*Penaeus monodon*) culture ponds, resulted in an improved water quality with less organic matter, promoted growth and survival rates and increased the health status of the cultured shrimp. Furthermore, (Wang *et al.*, 2005) demonstrated that the use of commercial probiotics (such as *Bacillus* spp., *Saccharomyces cerevisiae*, *Nitrosomas* spp. and *Nitrobacter* spp.) in white-leg shrimp (*Litopenaeus vannamei*) cultures improved water quality parameters, like enhancement of organic matter decomposition, and reduction in nitrogen and phosphorus concentrations in the rearing water.

2.4.4.5. Stress tolerance

The effects in aquaculture of probiotics on stress tolerance are a new field of investigation and a new proposed mechanism of action. One of the first studies regarding this thematic was developed by (Carnevali *et al.*, 2006), which detected significant lower levels of cortisol in seabass fed with a bacterial probiotic strain (*Lactobacillus delbrueckii* subsp. *delbrueckii*). Stress factors can trigger cortisol plasma levels, which can be harmful to the immune system and detrimental for the animal's welfare. Furthermore, as in mammals, cortisol can act as a proteolytic and catabolic agent, delaying somatic growth. Hence, cortisol could be considered as a good parameter to assess the animal's welfare (Carnevali *et al.*, 2006).

2.4.5. Probiotics' origin

The selection of a specific probiotic (single strain or mixed culture of multistrains/multispecies) for a specific host is a critical decision since probiotics can often demonstrate a host specific and strain specific different modes of action. Additionally, an inappropriate microorganism can not only fail the colonization process and its establishment in the gastrointestinal tract, but it can also lead to undesired effects on the host. It is widely accepted that probiotics from autochthonous sources, same species indigenous microbiota or even from the natural environment, could be the best and more advantageous approach. Furthermore, the indigenous microbiota is regarded as a key part of an animal's healthy status, standing as an important component of the mucosal barrier, which represents the first line of defence. The associated advantages include better tolerance to the gastrointestinal tract, greater chance to compete successfully with pathogens, greater chances of becoming predominant and to persist in the gut environment after withdrawal (Verschuere *et al.*, 2000a; Balcázar *et al.*, 2007a; Nayak, 2010; Sun *et al*., 2013; Pérez-Sánchez *et al.*, 2014). Nevertheless, the use of probiotics isolated from fish species different from the host species has also been extensively conducted. For instance, (Díaz-Rosales *et al.*, 2009) demonstrated that the use of two probiotic strains (*Shewanella putrefaciens* Pdp11 and *Shewanella baltica* Pdp13), both previously isolated from gilt-head seabream, as feed supplement for 60 days, increased the growth rate of the Senegalese sole and significantly increased the survival rate against the fish pathogen *Photobacterium damselae* subsp. *piscicida*. Likewise, (Díaz-Rosales *et al.*, 2009) reported that a bacterial strain isolated from gilt-head seabream, identified as *Vagococcus fluvialis*, improved the survival rate of seabass when challenged with *Vibrio (Listonella) anguillarum*. At the same time, the use of commercial probiotics, that have been mostly developed for terrestrial animals or humans, have also been applied to aquaculture (Nayak, 2010). For example, (Castex *et al.*, 2008) concluded that the use of Bactocell®, an authorized commercial probiotic for aquaculture containing *Pediococcus acidilactici* MA18/5M (with QPS status) previously authorized by the EU and successfully used in broiler chicken and fattening pig, also had a positive effect improving the food conversion ratio and the survival rate of western blue shrimp (*Litopenaeus stylirostris*), when challenged with vibriosis.

2.4.6. Microbial groups assessed as probiotics in aquaculture practice

The use of probiotics, both in human and veterinary medicine, has generally implied and relied on the use of Gram-positive bacteria, mainly LAB. Similarly, a major part of the probiotics proposed for aquaculture belong to the group of LAB. Nonetheless, the use of other microorganisms has been proposed. Therefore, the use of probiotics comprises microorganisms such as Gram-positive and Gram-negative bacteria, and yeasts (Naidu *et al.*, 1999; Newaj-Fyzul *et al.*, 2014; Pérez-Sánchez *et al.*, 2014).

2.4.6.1. Gram-positive bacteria

In aquaculture, the use of probiotics has been centred on the use of LAB (mainly belonging to the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Enterococcus*, *Carnobacterium* and *Pediococcus*) (see section 5), as well as on other Gram-positive bacteria, primarily belonging to the genus *Bacillus*, that has been gaining popularity since the late 90's (Balcázar *et al.*, 2006; Wang *et al.*, 2008a; Cutting, 2011; Newaj-Fyzul *et al.*, 2014; Pérez-Sánchez *et al.*, 2014). Bacteria belonging to the genus *Bacillus* constitute a diverse group of rod-shaped, Gram-positive bacteria with the ability to form endospores in adverse environments (Pérez-Sánchez *et al.*, 2014). Most *Bacillus* are not harmful for humans nor animals, besides that, they are important producers of secondary metabolites, such as antibiotics and enzymes and have seen a considerable success in aquaculture. Multiple species of *Bacillus* have been assessed as probiotics, like *B. subtilis*, *B. claussi*, *B. cereus*, *B. licheniformis* and *B. coagulans* (Cutting, 2011; Newaj-Fyzul *et al.*, 2014). The potential associated with the genus *Bacillus* and the increasing interest in it has led to several studies assessing its characteristics and abilities and pointing towards its benefits in aquaculture, which include improvements in nutritional parameters, survival rate against pathogens and even water quality as stated before (Dalmin *et al.*, 2001; Balcázar *et al.*, 2007b; Wang *et al.*, 2008a; Merrifield *et al.*, 2010b). For instance, (Balcázar *et al.*, 2007b) reported a significant improvement of the survival rate of white-leg shrimp when challenged with *Vibrio parahaemolyticus*, using a feed supplementation with *B. subtilis*. Besides LAB and *Bacillus* spp. other Gram-positive have been assessed and used in trials as probiotics in aquaculture. For example, in (Sharifuzzaman *et al.*, 2011) the use of cellular components of a strain isolated from the intestine of rainbow trout and identified as *Rhodococcus* SM2, not only stimulated the immune response but

also significantly reduced the mortality rate of rainbow trout, after being challenged with *Vibrio (Listonella) anguillarum.*

2.4.6.2. Gram-negative bacteria

The range of Gram-negative bacteria evaluated and used as probiotics in aquaculture is wide, being the most common species *Aeromonas*, *Enterobacter*, *Pseudomonas*, *Shewanella* and *Vibrio* (Nayak, 2010; Newaj-Fyzul *et al.,* 2014). For instance, after previously performing, with success, an *in vitro* trial to show the pathogen's growth inhibition, (Gram *et al.,* 1999) reported that the use of a *Pseudomonas fluorescens* strain reduced the mortality rate of rainbow trout when facing a pathogenic strain of *Vibrio (Listonella) anguillarum*. Similarly, (Irianto and Austin, 2002b) showed the efficacy of *Aeromonas hydrophila* and *Vibrio fluvialis* in controlling *Aeromonas salmonicida* infection in rainbow trout. Moreover, (Ström-Bestor and Wiklund, 2011) reported that a *Pseudomonas* sp. isolate MSB1 inhibited, *in vitro* conditions, the growth of multiple RTFS agent serotypes (*Flavobacterium psychrophilum*), with the likely production of siderophores as a mechanism of action, resulting in an iron deficiency in the supernatant, suggesting that future *in vivo* experiments should assess its use as probiotic in rainbow trout aquaculture. The use of bacteria belonging to genera with potential fish pathogenic species is common in aquaculture (such as *Aeromonas, Pseudomonas, Vibrio*), nevertheless, its safety should always be assessed, including the presence of resistance determinants or other virulence factors, in order to assure its safety to humans and animals (Leyva-Madrigal *et al.,* 2011; Muñoz-Atienza, 2015).

2.4.6.3. Yeasts

Yeasts are ubiquitous microorganisms, being a widespread part of the aquatic microbiota systems. Furthermore, yeasts can easily adhere to fish intestinal mucus and colonize their gastrointestinal tract, as it has been demonstrated in rainbow trout by Andlid (Andlid *et al.,* 1995). These features pose them as good potential probiotics, with low risk of harmful invasions associated (Gatesoupe, 2007). The main species of yeasts, which gather the most interest associated with probiotic potential in aquaculture are *Saccharomyces cerevisiae* and *Debaryomyces hansenii* (Pérez-Sánchez *et al.,* 2014). For instance, (Sheikhzadeh *et al.,* 2012) demonstrated that the use of a commercial

preparation containing *Saccharomyces cerevisiae*, as a feeding supplement for 50 days, caused a significant increase on the growth performance of rainbow trout, as well as an increase of skin non-specific immune parameters, namely enzymatic activities (*e.g.*, lysozyme activity) of the skin mucus. Likewise, (Tovar-Ramírez *et al.,* 2004; Reyes-Becerril *et al.,* 2008) also demonstrated the benefits of using *Debaryomyces hansenii* in aquaculture. It was also demonstrated that a yeast-based supplementation, for 4 weeks, significantly stimulated the cellular innate immune parameters of gilt-head seabream, and that it also regulated the mRNA expression of immune-associated genes, especially in haematopoietic organs (Reyes-Becerril *et al.,* 2008).

2.5. Lactic Acid Bacteria (LAB)

2.5.1. Properties and taxonomy of LAB

The concept of LAB comprises a wide heterogenous group of genera, which includes several species in it. They form a wide group of asporogenous Gram-positive rods and cocci bacteria that share morphological, metabolic, and physiological characteristics. LAB are usually characterized as aerobic to facultatively anaerobic bacteria, oxidase, catalase (lack of catalase *sensu stricto*) and benzidine negative, and that also lack cytochromes. Additionally, LAB are also chemoorganotrophic and fermentative bacteria, which have lactic acid as the major end product of carbohydrate's (*e.g.* glucose) fermentation (Klein *et al.,* 1998; Cintas *et al.,* 2001; Carr *et al.,* 2002;). According to current taxonomic classifications, the LAB group includes, among others, bacteria belonging to the genera: *Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus sensu stricto, Tetragenococcus*, *Vagococcus* and *Weisella* (Stiles and Holzapfel, 1997; Cintas *et al.*, 2001; Claesson *et al.,* 2007).

LAB are, from a nutritional perspective, fastidious, since they require multiple available nutrients such as carbohydrates, amino acids, peptides, nucleic acid derivatives and vitamins. Nevertheless, they are quite widespread in different environments. They are commonly found in nutritionally rich substrates such as milk and other dairy products, fermented foods (meats, vegetables, bread), vegetables and fruits (even when rotting), and silage. Besides foods, LAB can also be found in the gastrointestinal tract and other mucous membranes of animals and humans, due to their ability to adhere to mucosal

surfaces, which grants them a competitive advantage (Aguirre and Collins, 1993; Naidu *et al.*, 1999, Gómez-Sala *et al.*, 2019).

2.5.2. Applications of LAB

2.5.2.1. Lactic acid bacteria and food

Food preservation techniques, namely fermentation, can be traced back to the dawn of our civilization, far as 6000 BC, a time when humankind was starting to domesticate plants and animals. It is estimated that the process of making cheese can actually be traced back as 8000 years in the fertile crescent area in the Middle East. Back then, the process was obviously purely empirical, with no awareness of the role played by microorganisms. In the decade of 1850 microbiology flourished, and little after, in 1861, pasteurization was developed, and for the first time, the role of microorganism in fermentation was acknowledged. That discovery led to a large-scale development of fermentation processes applied in fermented foods and alcoholic beverages, with LAB standing out in a variety of dairy, vegetables, meat, and fish fermentation processes. The primary purpose of fermentation was to achieve a preservation effect on food, with an extended shelf-life and an improvement on food hygiene and safety. Currently, and due to the technological advancements that outcompete the traditional fermentation processes, these products are also praised due to their sensorial and organoleptic characteristics (Caplice and Fitzgerald, 1999; Lücke, 2000; Ross *et al.*, 2002; Gómez-Sala *et al.*, 2019). Therefore, the term biopreservation has surged as the linking piece between food preservation and these microorganisms, or their metabolites (Lücke, 2000). Depending on the main purpose of their metabolic activity, live cultures can be classified either as starter cultures, which have as main purpose a technological action (like flavour, aroma and texture) and have antimicrobial activity as a secondary one; or protective cultures, which have the antimicrobial activity as their primal function. Either way, starter and protective cultures can actually be the same culture, only applied for different purposes, under different conditions (Holzapfel *et al.*, 1995; Stiles, 1996; Cintas *et al.*, 2001; 2011; Vermeiren *et al.*, 2004). Moreover, fermentation has several roles in food processing: i) dietary enrichment, creating a variety of flavours, aromas and textures in food; ii) preservation through lactic acid, acetic acid, alcoholic, and other fermentations; iii) bio-enrichment of food substrates with vitamins, proteins, essential amino acids and fatty acids; iv)

detoxification; and v) decrease in cooking times and fuel requirements (Steinkraus, 2002). LAB, as for the bacteria and for their metabolic products, have a significant role in food preservation. They can cause microbial interference to food spoilage and pathogenic bacteria through several mechanisms (further developed in section 6). In southern Europe, for instance, the most used starter cultures for meat fermentation are LAB species such as *Lactobacillus sakei, Lactobacillus curvatus* and *Lactobacillus plantarum* (Hugas, 1998; Gómez-Sala *et al.,* 2019).

Nevertheless, the uncontrolled growth of some LAB, like *Leuconostoc* spp. and *Lactobacillus sakei*, can cause spoilage in some food and food products, like meats. They have been associated with the production of slime in processed meats, and sulphide-producing strains of *Lactobacillus sakei* have been associated with the spoilage of vacuum packaged meats (Hugas, 1998). Additionally, some LAB like *Enterococcus* spp. have the ability to produce biogenic amines (*e.g.*, histamine, tyramine) by means of decarboxylase enzymes, in cheese and fermented sausages. When ingesting a large amount of these biogenic amines, it can lead to food intoxication, even when used as a starter culture. This can cause symptoms such as headache, vomiting, increased blood pressure and stronger allergic reactions, especially when considering more sensitive people (Giraffa, 2002).

2.5.2.2. LAB as probiotics for humans and animals

Probiotics used in human and terrestrial animals have, for decades, been centred on the use of bacteria belonging to the genus *Bifidobacterium* and to the group of LAB, namely, but not only, belonging to the genera *Lactobacillus* and *Enterococcus.* Likewise, LAB have been the major representatives of probiotics, whether regarding food (historically dairy products), pharmaceutical products, or even feed additives for animals (Irianto and Austin, 2002a; Holzapfel and Schillinger, 2002; Ljungh and Wadström, 2006; Balcázar, 2007b; Cintas *et al.,* 2011; Pérez-Sánchez *et al.,* 2014). The importance and relevance of LAB as probiotics is mostly due to their consideration, in general, as safe microorganisms under the classification of Generally Recognized as Safe (GRAS, by the Food and Drugs Administration) and under the European QPS status (Cintas *et al.,* 2001; 2011; EFSA, 2005a; 2005b; 2007; Gómez-Sala *et al.,* 2019).

2.5.2.3. LAB as probiotics in aquaculture

The growing interest in the use of probiotics in aquaculture is followed by a growing interest and greater comprehension of the application of LAB to aquaculture, since most probiotics proposed as biocontrol agents in aquaculture belong to that group (Verschuere *et al.*, 2000a; Gatesoupe, 2008). This interest in LAB is mostly due to: i) most LAB being regarded as safe microorganisms, both for humans and animals (GRAS status and/or QPS status); ii) several LAB strains being currently legally accepted as probiotics for humans and animals; iii) a strain of *Pediococcus acidilactici* being currently authorized in the EU to be used in aquaculture; and iv) the acknowledgment that several LAB (*Lactococcus, Lactobacillus, Leuconostoc, Carnobacterium, Enterococcus, Vagococcus,* among others) are part of the normal intestinal microbiota of fish, namely freshwater fish (Ringø and Gatesoupe, 1998; Cintas *et al.*, 2001; Austin, 2002; Ljungh and Wadström, 2006; EFSA 2005a; 2005b; 2007; 2019; Pérez-Sánchez *et al.*, 2014; Commission Implementing Regulation EU 2020/151).

As previously stated, the main mechanisms of action performed by LAB include competition for nutrients, production of organic acids (lactic and acetic acid), and production of compounds with antimicrobial activity, such as hydrogen peroxide, CO_2, diacetyl, acetaldehyde, D-isomers of amino acids, reuterin and bacteriocins (Cintas *et al.*, 2001; Cotter *et al.*, 2005). Additionally, LAB have shown several probiotic beneficial effects on fish, such as improvement of the survival rate against pathogens, modulation of the immune system, growth stimulation and improvement of water quality (Verschuere *et al.*, 2000a; Balcázar *et al.*, 2006; 2008; Wang *et al.*, 2008a; Pérez-Sánchez *et al.*, 2014).

Multiple LAB have been assessed as probiotics in aquaculture, with the genera *Enterococcus, Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Vagococcus* being among the most studied ones (Araújo, 2015). For instance, (Balcázar *et al.*, 2007a) demonstrated that rainbow trout supplemented with three LAB probiotic strains (*L. lactis* subsp. *lactis* CLFP 100, *Leuconostoc mesenteroides* CLFP 196 and *Lactobacillus sakei* CLFP 202) had a significant increase in the phagocytic activity of head kidney leucocytes and in the alternative complement activity in the serum. Furthermore, it was demonstrated that rainbow trout fed with those probiotics had a higher survival rate when challenged with *A. salmonicida* subsp. *salmonicida*, compared to the control group. Additionally, (Vendrell *et al.*, 2008) demonstrated that the oral administration, for 30 days, of *Leuconostoc mesenteroides* CFLP 196 and *Lb. plantarum* CLFP 238, isolated from

salmonids, significantly diminished the mortality rate of rainbow trout after challenged with *L. garvieae*. More recently, (Araújo *et al.*, 2015a) demonstrated that several *L. lactis* strains, isolated from rainbow trout and respective rearing environment, exerted antimicrobial activity towards four pathogenic strains of *L. garvieae*, demonstrating that both rainbow trout and rearing environment can work as potential sources of LAB with probiotic potential towards pathogens. At the same time, some LAB that are usually used as probiotics in humans have been tested in fish as well. For instance, (Nikoskelainen *et al.*, 2001b) demonstrated that a LAB intended for human use (*Lactobacillus rhamnosus* ATCC 53103) effectively decreased the mortality rate of rainbow trout after being challenged with *A. salmonicida* subsp. *salmonicida*. Since the probiotic strain used is considered safe for humans, it was suggested that, after those positive results in rainbow trout, the strain could be a promising probiotic for fish without associated risks for human consumption. In human trials, *Lb. rhamnosus* ATCC 53103 (also known as *Lb. rhamnosus* strain GG) was associated with prevention and treatment of acute diarrhoeas and antibiotic-induced diarrhoeas, prevention of cow milk-induced food allergy in kids and traveller's diarrhoea, among other beneficial effects (Goldin and Gorbach, 2008). Likewise, (Chang and Liu, 2002) demonstrated that the use of *Enterococcus faecium* SF68, a LAB probiotic associated with prevention of acute and antibiotic-induced diarrhoeas in human medicine, improved the survival rate of eel infected with *Ed. tarda*. Similarly, LAB probiotics isolated from other animals have also been assessed as potential probiotics for aquaculture. For instance, (Wang *et al.*, 2008b) showed that the use of *E. faecium* ZJ4, isolated from a piglet, improved nutritional parameters (like final weight and daily weight gain), and increased myeloperoxidase and respiratory burst activities, in treated Nile tilapias (*Oreochromis niloticus*).

2.5.3. Antimicrobial activity of LAB

Regardless of being applied for food technological functions, protective cultures, or as probiotics, LAB possess the ability to inhibit the growth of a wide range of microorganisms, both food (spoilage and pathogenic) and clinically related ones. Their primary antimicrobial activity is due to competition for nutrients, formation of organic acids (mainly lactic and acetic acid, which lower the pH and thereby acidify the substrate), and production of antimicrobial compounds. These antimicrobial compounds can range from ethanol, CO_2, diacetyl, acetaldehyde, hydrogen peroxide, D-isomers of certain

amino acids, reuterin and bacteriocins (further developed in section 6) (Caplice and Fitzgerald, 1999; Cintas *et al.*, 2001; Ross *et al.*, 2002; Deegan *et al.*, 2006; Gómez-Sala *et al.*, 2019).

Out of all antimicrobial abilities and compounds of LAB, bacteriocins produced by them have attracted over the past years a vast interest and attention in multiple research areas, which is still an expanding process. Bacteriocins have a wide potential and are currently used in several fields, ranging from food technology and biopreservation, to probiotic utility through inhibition of pathogenic bacteria (both in human and veterinary medicines), and even anti-carcinogenic potential (Klaenhammer, 1993; Cintas, *et al.*, 2001; Cotter *et al.*, 2005; Lancaster *et al.*, 2007; Nishie *et al.*, 2012; Yang *et al.*, 2014).

2.6. Bacteriocins

Bacteriocins are an heterogenous group of ribosomally synthesized peptides or proteins produced by bacteria, with or without post-translational modifications, that exert antimicrobial activity towards other bacteria (Klaenhammer, 1993; Cintas *et al.*, 2001; 2011; Cotter *et al.*, 2005; Yang *et al.*, 2014).

It is estimated that a wide range of bacteria (both Gram-positive and Gram-negative) can produce at least one bacteriocin, nevertheless, most of the bacteriocins so far discovered are produced by Gram-positive bacteria, particularly LAB ones. Due to their variety and expanding discovery, an open-access database, BACTIBASE, was built to compile the ever-growing number of known and described bacteriocins (Klaenhammer, 1993; Cotter *et al.*, 2005; Nishie *et al.*, 2012).

Being an heterogenous group, bacteriocins vastly vary in physico-chemical properties, antimicrobial spectrum, mode of action, biosynthesis, molecular mass, structure, transport and regulation of their production, among many other variables (Cintas *et al.*, 2001; 2011; Nishie *et al.*, 2012).

2.6.1. Classification of bacteriocins

Despite being an heterogenous group of proteinaceous compounds, LAB-produced bacteriocins still share common traits that allow them to be classified into a system of distinct classes. LAB bacteriocins belonging to class I and II are by far the most studied ones, mostly due to the fact of being the most abundant and the ones with most industrial

and commercial potential (Klaenhammer *et al.,* 1993; Nes *et al.,* 1996; Cintas *et al.,* 2001).

Class I bacteriocins, also known as lantibiotics (divided into type A and B), are small heat-stable polycyclic peptides (molecular mass <5 kDa), that contain unusual and post-translationally modified amino acids. Unique characteristics of these bacteriocins include the presence of intramolecular rings of thioether amino acids called lanthionine and β-methyl-lanthionine, which ultimately contributes to their classification as lantibiotics (lanthionine-containing antibiotics). Additionally, another post-translational common modification is the presence of dehydrated amino acids, such as dehydroalanine and dehydrobutyrine. The condensation between them and neighbouring sulfhydryl groups of cysteine residues form the intramolecular rings previously mentioned (Nes *et al.,* 1996; Moll *et al.,* 1999; Guder *et al.,* 2000; Cintas *et al.,* 2001, Zendo *et al.,* 2010).

Type A lantibiotics are elongated, flexible and cationic, they interact with the bacterial membranes through pore formation. Nisin (further developed in section 7) is an example of a type A lantibiotic, and it is perhaps the most characterized and well-studied bacteriocin to date. Furthermore, nisin is to date the only bacteriocin internationally approved as food additive. Type B lantibiotics have a globular structure, they can either be neutrally charged or anionic, and are enzyme inhibitors and immunologically active peptides (Moll *et al.,* 1999; Guder *et al.,* 2000; Cintas *et al.,* 2001; 2011).

Class II bacteriocins (non-lantibiotics) are small (molecular mass < 10 kDa), heat stable peptides, usually anionic and amphiphilic, that are not exposed to intensive post-translational modifications, and that act mainly through membrane permeabilization provoking the leakage of cellular content. Class II bacteriocins can be further divided into four subclasses (Moll *et al.,* 1999; Cintas *et al.,* 2001; Cotter *et al.,* 2005; Nissen-Meyer *et al.,* 2009, Zendo *et al.,* 2010). Class IIa bacteriocins (pediocin-like bacteriocins) have a strong antimicrobial activity towards a broad range of Gram-positive spoilage and food-borne pathogens, namely *Listeria monocytogenes*, responsible for recent and recurring episodes of listeriosis. The study of antilisterial LAB has led to a broad description and characterization of multiple Class IIa bacteriocins, synthetized by multiple *Pediococcus, Leuconostoc, Enterococcus, Lactobacillus* and *Carnobacterium* strains (Cintas *et al.,* 2001; Nissen-Meyer *et al.,* 2009). This subclass is referred as the pediocin-like subclass due to the bacteriocin pediocin PA-1 (produced by several strains of *Pediococcus acidilactici*) , the most characterized bacteriocin within the group, produced, for instance, by multiple *Pediococcus acidilactici* strains of meat origin and others (Pucci *et al.,* 1988;

Henderson *et al.*, 1992; Marugg *et al.*, 1992; Cintas *et al.*, 1998a; 2001). While most bacteriocins display full activity by themselves, some bacteriocins require a complementary action of two peptides to achieve full activity, like those belonging to the subclass IIb (two-peptide bacteriocins) (Cintas *et al.*, 1998b; 2001; Nissen-Meyer *et al.*, 2009). In some class IIb bacteriocins, the two peptides separately do not possess antimicrobial activity by their own, only when combined simultaneously (*e.g.*, lactococcin G and M); nevertheless, there are some other class IIb bacteriocins where the peptides have some antimicrobial activity independently, achieving an enhanced activity when combined, which is greater than the additive effect of them when separated (Cintas *et al.*, 1998b). The class IIc bacteriocins, also known as sec-dependent bacteriocins, may share significant homology with other class II bacteriocins. However, the exportation of class IIc bacteriocins occurs via the sec-dependent pathway, unlike the other bacteriocins, which rely on a dedicated transport system. Class IIc bacteriocins include: divergicin A, acidocin B, enterocin P, bacteriocin 31 and listeriocin 743A (Cintas *et al.*, 2001; Kalmokoff *et al.*, 2001). Lastly, the class IId have been proposed to include class II bacteriocins that do not fit in any of the previous subclasses, as for instance, the enterocins L50 isolated from Spanish-dry fermented sausages (Cintas, 1995; Cintas *et al.*, 1995; 1998b; 2001).

Class III bacteriocins are non-lantibiotic, large (>30 kDa), heat-labile bacteriocins. They are inactivated when exposed to heat treatment (60-100ºC, for 10-15 minutes). Examples of bacteriocins belonging to this class are helveticin J and enterolisin A (Cintas *et al.*, 2001; Kemperman *et al.*, 2003).

Throughout the years, the classification system has not been consensual (Heng and Tagg, 2006). Therefore, alterations and even new types of organizational classifications have been proposed. (Kemperman *et al.*, 2003) suggested the creation of a new class for circular bacteriocins, such as circularin A, microcin J25, or gassericin A since they do not fit in any of the previous classes. They are ribosomally synthetized, post-translationally modified, small-sized bacteriocins (which excludes them from class II and III), which also do not contain modified amino acids, therefore excluding them from class I too. On the other hand, (Cotter *et al.*, 2005) proposed deeper adjustments by reclassifying all labile compounds of large size (typically belonging to class III) as bacteriolysins (murein hydrolases), excluding them from the classical classification as bacteriocins since they differ in large extent from the classic definition. It was proposed the creation of two large classes, being the first class reserved for lantibiotics, and the second one reserved for all

non-lanthionine-containing bacteriocins (including the circular ones), excluding bacteriolysins from it. More recently, (Zouhir *et al.*, 2010) recommended a new classification based on structure characteristics, grouping bacteriocins under 12 new groups.

2.6.2. Physico-chemical properties

Most of the small-sized bacteriocins (class I and II) share some features, such as being highly cationic at pH 7.0, a high isoelectric point, presence of hydrophobic and/or hydrophilic regions (being amphiphilic when both of them are present). These type of bacteriocins are also active at acidic and physiological pH values. LAB bacteriocins also tend to have thermostability, although factors such as the purification stage of them can highly influence it. Usually, bacteriocins in cell-free supernatants resist to autoclaving and heating processes (100-121ºC). Nevertheless, thermal stability can be lost in some bacteriocins when partially purified or purified to homogeneity. Nonetheless, nisin is an exception, because even when purified it remains active after heating at 100ºC for 10 minutes at pH of 2.0 (Cintas, 1995; Nes *et al.*, 1996; Moll *et al.*, 1999; Cintas *et al.*, 2001).

2.6.3. Antimicrobial spectrum of LAB bacteriocins

According to Tagg *et al.* (1976), most bacteriocins of Gram-positive bacteria exhibit inhibitory activity towards a wide range of Gram-positive bacteria. Moreover, most LAB-bacteriocins exert bactericidal activity beyond closely related species and beyond ecological niches (Klaenhammer, 1993). According to Jack *et al.* (1995), some general observations can be made regarding the antimicrobial activity of class I and class II bacteriocins: i) within a given species, some strains might be sensitive and others might be resistant to a particular bacteriocin; ii) a strain that appears to be sensitive to a bacteriocin may also have some cells in the population that are resistant to it; iii) a strain can be sensitive to a bacteriocin while being resistant to a similar type of bacteriocin; iv) cells of a strain producing one bacteriocin can be sensitive to another bacteriocin; v) although the spores of a strain, whose cells are sensitive to a bacteriocin, are resistant to that bacteriocin, they become sensitive after germination; and vi) under normal conditions, Gram-negative bacteria are not sensitive to bacteriocins produced by LAB. Nevertheless, it is relevant to state that in the presence of certain chemical agents (*e.g.*,

EDTA, organic acids, other chelants) or stressing environmental conditions (*e.g.*, pH, freezing, mild heating and high hydrostatic pressure), which weaken cell integrity, some LAB bacteriocins, like nisin, exert activity towards several species of Gram-negative bacteria (Stevens *et al.*, 1991; Kalchayanand *et al.*, 1992; Jack *et al.*, 1995; Cintas *et al.*, 2001).

To sum up, the antimicrobial spectrum of bacteriocins can be organized into three distinct groups: i) bacteriocins with a narrow spectrum of activity, restricted to the same species of the producing strain or to species of the same genera; ii) bacteriocins with intermediate antimicrobial spectrum, inhibiting other genera of LAB and Gram-positive bacteria, including food borne pathogens like *Ls. monocytogenes*, *Clostridium perfringens* and *Clostridium botulinum*; lastly iii) bacteriocins with a broad spectrum of activity, including several Gram-positive bacteria, which is the group where nisin fits (Cintas *et al.*, 2001; 2011).

2.6.4. Purification of bacteriocins

Considering that bacteriocins are proteinaceous products excreted to the extracellular medium, most purification protocols developed start with the concentration of the cell-free supernatant of the bacteriocin producer culture, therefore reducing the volume of the sample. Since bacteriocin yields decrease during the overall process, it is recommendable to optimize the production by adjusting experimental conditions (*e.g.*, medium composition, temperature, pH, time, etc.) to the optimal ones of the respective bacteriocin (Venema *et al.*, 1997; Cintas *et al.*, 2001; 2011).

Among the most widely used strategies to perform the concentration step are: i) filtration by dialysis or ultrafiltration (ten Brink *et al.*, 1994; Parente and Ricciardi, 1999;); ii) protein precipitation by using salts, like ammonium sulphate (Holo *et al.*, 1991); iii) protein extraction with organic solvents, like butanol (Piva and Headon, 1994); iv) vacuum-drying; and v) lyophilization (Cintas *et al.*, 2001). However, in order to obtain higher purity, it is necessary to separate the bacteriocins from other proteinaceous compounds using techniques based on their physico-chemical properties (Casaus, 1998).

One of the most successful purification protocols conceived for bacteriocins of LAB origin was initially formulated by (Nissen-Meyer *et al.*, 1993). It takes into consideration the overall low molecular weight, cationic nature, and relative hydrophobicity of those bacteriocins (Cintas *et al.*, 2001).

This method consists of a succession of steps that usually include: i) growing the producing strain in a suitable nutrient medium under optimal conditions for bacteriocin production; ii) removing cells by centrifugation and protein precipitation from the cell-free supernatant through addition of ammonium sulphate; iii) sequential fractionation of the bacteriocins through multiple chromatographic steps, which include cation-exchange, hydrophobic interaction chromatography and reversed-phase fast-protein liquid chromatography (FPLC) (Cintas *et al.,* 2001). This procedure has been applied to purify multiple lantibiotics, like nisin A (Cintas, 1995; Cintas *et al.,* 1998a), and also non-lantibiotics, such as enterocins L50 (Cintas *et al.,* 1998a; 1998b) or enterocin A (Herranz *et al.,* 2001), among many others.

2.7. Nisin

2.7.1. Background, utilization and properties

Nisin is a 34-residue-long peptide with antimicrobial activity and the most well characterized and described bacteriocin of the lantibiotic class. Its history can be traced back to the late 1920s. In 1928, Rogers and Whittier described for the first time the inhibitive activity of certain lactococcal strains towards other LAB starter cultures in dairy products. In 1933, (Whitehead, 1933) also described the existence of a proteinaceous compound with antimicrobial activity, which later led to the description of that same compound as nisin (firstly as group N inhibitory substance), by Mattick and Hirsch (1944; 1947). In 1953, England was the first country to allow the commercialization and use of nisin, and since then more than 50 countries around the globe have approved its use. In 1969, FAO/WHO assessed it to be safe for food, and in 1983 nisin was listed as a legal food additive by the EU (as E234), lastly, in 1988 the FDA approved its use too (timeline scheme available in *Figure 2.8*) (Delves-Broughton *et al.,* 1990; Cotter *et al.,* 2005;). Since then, nisin has been widely used as a legal food preservative, with relative success in dairy products and canned food, showing, for example, antilisterial properties, ability to inhibit vegetative cells of *Bacillus* spp. and *Clostridium* spp., and to prevent spore outgrowth. Additionally, nisin is also a bacteriocin with promising pharmaceutical and clinical potential. Not only nisin preparations have been successfully used in bovines as an anti-mastitis compound, but also nisin has shown potential as an aquaculture probiotic, for instance, as an agent in rainbow trout trials

(Delves-Broughton *et al.*, 1990; Abee *et al.*, 1995; Cintas *et al.*, 1998a; 2001; Araújo *et al.*, 2015b).

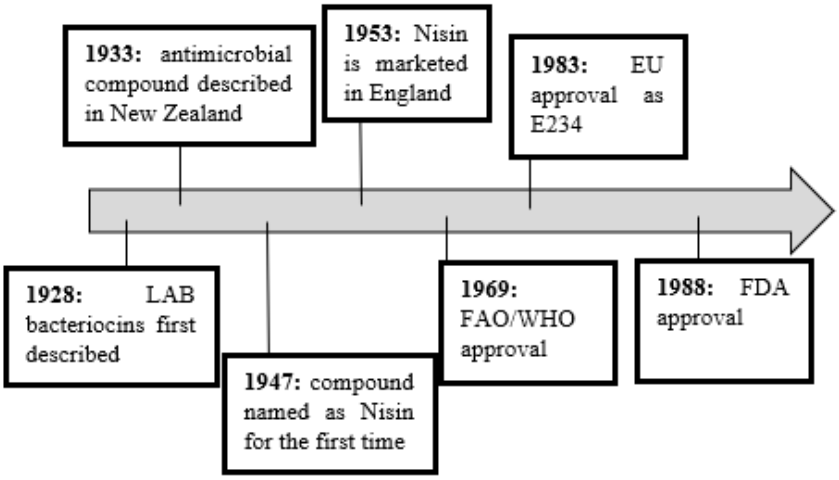

Figure 2.8. Timeline of the discovery and commercialization of nisin worldwide.
Adapted from: Cotter et al. (2005).

Nisin is a class Ia (lantibiotic) bacteriocin, consisting of 34 amino acids in a screw-shaped conformation. Being a class I bacteriocin, nisin is post-translationally modified, its serine and threonine residues are dehydrated into dehydroalanine and dehydrobutyrine, respectively. Five of these dehydrated residues couple with cysteines, forming thioether bonds that produce the characteristic lanthionine rings (*Figure 2.9)* (Breukink and de Kruijff, 1999, Zendo *et al.*, 2010).

One of the most described LAB species, which is extensively used in the dairy industry and consumption, is *L. lactis*. Besides being regarded as safe for animal and human consumption (QPS status) and widely industry used, multiple *L. lactis* strains produce several bacteriocins, including various variants of nisin (Nisin A and its natural variants, such as Z, Q, F and U) (Flórez *et al.*, 2008; Zendo *et al.*, 2010; Araújo *et al.*, 2015b). Nisin A (NisA) is the most well characterized LAB bacteriocin, and the only one commercially approved and used as a food preservative, while Nisin Z (NisZ) is the most widely spread natural variant of NisA. Both solely differ in one amino acid, at position 27, with an exchange from histidine (His) in NisA to asparagine (Asn) in NisZ (*Figure 2.9*) (Mulders *et al.*, 1991; Araújo *et al.*, 2015b).

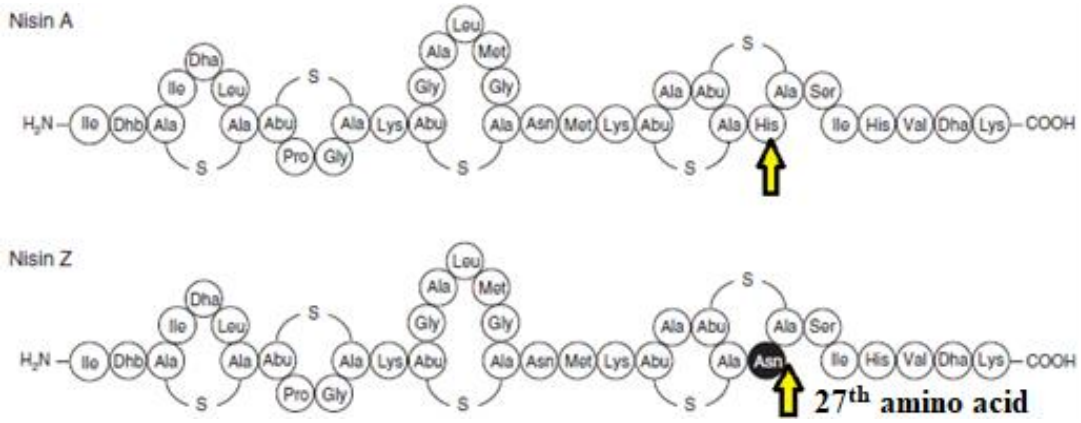

Figure 2.9. Comparative structure of NisA and NisZ, with yellow arrows pointing towards the 27th amino acid where histidine in NisA is substituted by asparagine in NisZ. *Adapted from: Horinouchi et al. (2010).*

Regarding the structure, nisin has two different clusters of residues. In the N-terminus it has a hydrophobic cluster, while in the C-terminus it has a hydrophilic one, which grants nisin amphipathic properties. This distribution of polar and apolar residues may be relevant concerning the mode of action and biological activity of nisin. The pH of the solution stands as a very influent factor on the solubility, stability and biological activity of nisin. Its solubility and stability decreases from optimal pH 2.0 to 6.0, being inactivated at neutral pH value (7.0). The stability of nisin is strictly related to its solubility, which is exemplified when at a pH of 2.0 nisin remains active after heating at 100ºC for 10 minutes. Additionally, the thioether rings formed, that account for the sulphur content of nisin, are thought to contribute to its functional properties (acidic mediums tolerance, thermostability, and specific bactericidal mode of action) (de Vuyst and Vandamme, 1994; Breukink and de Kruijff, 1999; Cintas *et al.*, 2001).

Nisin's molecular mass varies according to the natural variant in cause, for instance, NisA has a molecular mass of 3353 Dalton (Da), while NisZ has a molecular mass of 3330 Da (Guder *et al.*, 2000).

2.7.2. Antimicrobial spectrum of nisin

Nisin belongs to a strict group of bacteriocins which have a broad spectrum of activity. Nisin inhibits a large range of Gram-positive bacteria, mainly belonging to the genera *Actinomyces, Bacillus, Clostridium, Corynebacterium, Micrococcus, Lactobacillus, Lactococcus*, *Leuconostoc*, *Pediococcus*, *Pneumococcus*, and *Streptococcus*. Likewise, nisin has also demonstrated activity against other bacteria such as *Mycobacterium*

tuberculosis, *Staphylococcus pyogenes*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, or *Ls. monocytogenes*. Furthermore, nisin exhibits activity towards some Gram-negative bacteria, like *E. coli* or *S. enterica enterica* serotype typhimurium, whenever damage is induced to their outer membrane, such as a chemical damage done by EDTA, or organic acids, for instance (de Vuyst and Vandamme, 1994; Breukink and de Kruijff, 1999; Cintas *et al.,* 2001; Bauer and Dicks, 2005; Zendo *et al.,* 2010).

Moreover, nisin also reveals inhibitive activity towards spores, namely from the genera *Bacillus* and *Clostrydium* (Cintas *et al.,* 2001). Both NisA and NisZ are considered to have an identical antimicrobial spectrum of activity (de Vuyst and Vandamme, 1994).

2.7.3. Nisin's mode of action

Bacteriocins may act as bactericidal or bacteriostatic agents on sensitive cells, which can be highly influenced by multiple factors, dose, degree of purification, temperature, pH, to name a few. Most bacteriocins act in a bactericidal mode, although some bacteriocins can act as bacteriostatic, like lactocin 27, leucocin A-UAL187, and leucocin S (Cintas *et al.*, 2001).

Bactericidal bacteriocins mainly act through sensitive cell membrane's destabilization and permeabilization, which can be achieved by forming transitory pores or ionic channels in the cytoplasmatic membrane of sensitive cells (Abee *et al.,* 1995; Jack *et al.*, 1995; Bauer and Dicks, 2005).

Considering the above, it has been shown that nisin acts on sensitive cells through pore formation. The pore formation mechanism can be rationally divided in three steps: binding, followed by insertion into the lipid phase of the target membrane, and finally, the formation of the pores (Breukink and de Kruijff, 1999; Cintas *et al.*, 2001;).

Nisin preferably binds to membranes with greater amounts of anionic lipids, meaning that they are negatively charged membranes. Generally, Gram-positive bacteria contain relative higher concentration of anionic lipids in their plasma membrane, when compared with Gram-negative bacteria, which may partially justify the antimicrobial activity differences between those two groups (Breukink and de Kruijff, 1999)

Different regions of nisin take part during distinct stages of the process, for instance, the C-terminus part of nisin plays an important role on the initial step of the process, the binding step, by mediating the initial electrostatic interaction between the cationic charge of nisin and the anionic phospholipids of the membrane (Breukink and de Kruijff, 1999;

Cintas *et al.*, 2001). After binding, it is the insertion step, which depends on the amphiphilic properties of nisin. The N-terminus segment, the most hydrophobic part, is predominantly responsible for the insertion into the lipid phase of the membrane, therefore, relying on hydrophobic interactions. During the insertion step, the N-terminus is deeply inserted in the membrane, while the C-terminus is located close to surface. The peptide, on the overall, has a parallel orientation in respect to the membrane surface (Breukink and de Kruijff, 1999; Lins *et al.*, 1999; Bauer and Dicks, 2005).

Finally, after the insertion step, nisin may locally disturb the phospholipids, inducing a positive interface curvature. Afterwards, nisin recruits negatively charged lipids, creating a higher concentration of them in the surrounding zone. During this process, multiple peptides adopt a transmembrane orientation. Considering that the N-terminus is more deeply inserted, it is possible that the initiation of the pore formation leads to the translocation of the N-terminus to the inner leaflet of the membrane. This results in the formation of pores where the N-terminus is situated on the *trans* side of the membrane. Some elements of this process are still unknown, such as the active involvement of phospholipids in it. Nevertheless, the pore can eventually collapse during this final step, meaning that the whole peptide might translocate at that time. Once the pores are formed it will occur a rapid efflux of ions (potassium, inorganic phosphate), amino acids, glutamate, ATP, among other small substances. The increasing membrane permeability leads to the collapse of vital ion gradients and dissipation of the proton motor force (PMF) components (both transmembrane potential and pH gradient), which causes the cessation of biosynthesis processes and eventually leading to the cell death through termination of energy-requiring reactions (Driessen *et al.*, 1995; Breukink and de Kruijff, 1999).

Additionally, some *in vitro* tests showed that nisin also inhibited cell wall synthesis (Linnett and Strominger, 1973). Later this was found to be due to the formation of a complex between nisin and another molecule, called lipid II (Reisinger *et al.*, 1980). (Brötz *et al.*, 1998) demonstrated that nisin uses lipid II (which is a peptidoglycan precursor, crucial for the cell wall synthesis) as a docking molecule, for an eventual pore formation. During its cycle, lipid II is transported to the exterior side of the membrane, where it will eventually link with nisin. It is suggested that the N-terminus part of nisin, namely the two first rings, is essential for the binding between these two molecules, binding at the pyrophosphate part of the lipid II. Hence, nisin combines efficiently two lipid II-mediated mechanisms, one blocking lipid II from incorporation into peptidoglycan (therefore interfering with the cell wall synthesis), and secondly using lipid

II for targeted pore formation. This nisin-lipid II interaction potentiates the activity of nisin from the micromolar to the nanomolar range. Likewise, the presence of lipid II largely enhances the pore lifetime, becoming voltage-independent, and with a reduced anion-selectivity. When several nisin-lipid II complexes assemble they form a functional pore, where lipid II is an intrinsic part of the structure (Breukink and de Kruijff, 1999; Bauer and Dicks, 2005; Zendo *et al.,* 2010).

To summarize, nisin can permeabilize membranes through two distinct mechanisms, one targeted and one non-targeted. Whenever nisin interacts with lipid II, nisin not only forms a highly specific pore, but also interferes with the cell wall synthesis, working independently of a negatively charged surface. The diversity of nisin's mode of action makes it a greatly interesting molecule and an excellent candidate for new generation antimicrobial compounds (Bauer and Dicks, 2005).

2.7.4. Biosynthesis and regulation

Usually, bacteriocins are synthetized as biologically inactive precursors, that can be named as preprobacteriocins. The preprobacteriocin contains a C-terminal propeptidic domain extension (probacteriocin), and a N-terminal extension, that is cleaved off during the transport of the mature bacteriocin to the extracellular medium (Jack *et al.,* 1995; Nes *et al.,* 1996; Cintas *et al.,* 2001; 2011).

The biosynthesis of lantibiotics, such as nisin, is typically encoded by gene clusters. These gene clusters contain conserved genes that encode functions such as production, maturation, immunity and regulation. The particular biosynthesis of nisin is a complex process, that involves a cluster of 11 genes orderly arranged as *nis*A/Z BTCIP, *nis*RK, and *nis*FEG (Buchman *et al.,* 1988; Kuipers *et al.,* 1993; 1995; de Vos *et al.,* 1995; Siezen *et al.,* 1996; Cheigh and Pyun, 2005).

The *nis*A/Z gene encodes a NisA/Z precursor peptide of 57 amino acid residues, that possesses a 23-residue N-terminal leader part that is absent in mature form of nisin (*Figure 2.10*). The typical intracellular post-translational modifications of nisin involve the presence of membrane-associated peptides, which are encoded by *nis*B and *nis*C genes (Kuipers *et al.,* 1993; 1995; de Vos *et al.,* 1995; Cheigh and Pyun, 2005).

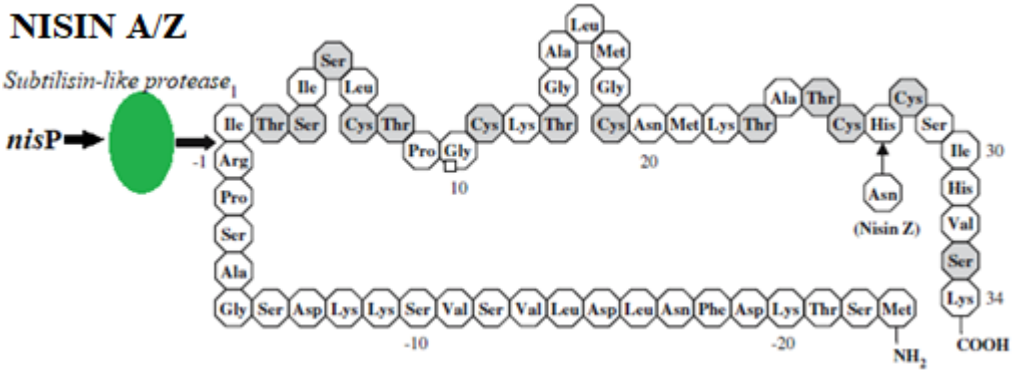

Figure 2.10. Structure of the nisin precursor peptide containing the N-terminal leader part. Action of the subtilisin-like protease (encoded by the gene *nis*P) cleaving the leader part. *Adapted from: Bauer and Dicks (2005).*

At some point, the modified nisin is finally translocated through the cytoplasmatic membrane by a transporter protein, of the ABC-transporters (Adenosine triphosphate Binding Cassette) family, which is encoded by the *nis*T gene (Qiao and Saris, 1996). Shortly after, or even during the translocation, the leader peptide is cleaved off in a proteolytic reaction, forming an extracellular mature nisin. The N-terminal leader peptide is removed by a subtilisin-like protease encoded by the gene *nis*P (van der Meer *et al.*, 1993). LAB that produce bacteriocins have mechanisms for self-protection (immunity) against the activity and toxicity of their own bacteriocins (Jack *et al.*, 1995; Cintas *et al.*, 2001;). Nisin-producing strains are no exception to this. In fact, there are two mechanisms by which nisin-producing strains achieve that self-protection: i) the *nis*I gene encodes a lipoprotein involved in it; and ii) the *nis*FEG gene, which is formed by the subunits *nis*F, *nis*E and *nis*G, encodes an ABC transporter active in nisin extrusion (Bauer and Dicks, 2005; Zendo *et al.*, 2010, Field *et al.*, 2019).

Nisin, as other lantibiotics, is produced in a growth-phase dependent process. Additionally, its biosynthesis is regulated by a two-component regulatory system, which comprises a sensor histidine kinase and a response regulator. Considering this, nisin acts as an auto-inducing peptide of its own regulation. The two genes involved in the two-component system belong to the initial nisin gene cluster: *nis*K and *nis*R. Firstly, a sensor histidine kinase, encoded by *nis*K, detects the extracellular auto-inducing signal. Subsequently, there is a response regulator, which is encoded by the gene *nis*R, that regulates the gene transcription of the gene cluster (Kuipers *et al.*, 1995; Ra *et al.*, 1996; Bauer and Dicks, 2005; Zendo *et al.*, 2010).

3. MATERIALS AND METHODS

The experimental part of this work was developed in the Grupo de Seguridad y Calidad de los Alimentos por Bacterias Lácticas, Bacteriocinas y Probióticos (SEGABALBP), Sección Departamental de Nutrición y Ciencia de los Alimentos (Nutrición, Bromatología, Higiene y Seguridad Alimentaria), Facultad de Veterinaria, Universidad Complutense de Madrid (UCM), Spain, from September 15 to December 15, 2019.

3.1. Bacterial strains and growth conditions

The bacterial strains used in this study belong to the SEGABALBP group bacterial collection and were appropriately stored in the Sección Departamental de Nutrición y Ciencia de los Alimentos (Nutrición, Bromatología, Higiene y Seguridad Alimentaria) of the Facultad de Veterinaria at the UCM. Among the strains used during this work was the target strain, RBT18, previously identified from the rainbow trout, as well as several fish pathogens used as indicator microorganisms. Some of the strains used as indicator microorganisms have been previously assessed as fish pathogens, like *L. garvieae* CF00021, *L. garvieae* JIP29-99 and *L. garvieae* CLG-4. The non-pathogenic bacterial strain *Pediococcus damnosus* CECT4797 was equally used as an indicator microorganism. Additionally, a previously identified NisA producing strain, *L. lactis* subsp. *lactis* BB24 (Cintas, 1995), as well as a NisZ producing strain, *L. lactis* subsp. *cremoris* WA2-67 (Araújo *et al.,* 2015a; 2015b), were used during this study as control parameters, regarding nisin production.

Throughout the study, all the bacterial strains mentioned above were aerobically grown in de Man, Rogosa and Sharpe broth (MRS, Oxoid Ltd., Basingstoke, UK) and incubated overnight at 30ºC, unless otherwise stated.

3.2. Strain RBT18 identification

The target strain of this study, named as RBT18, was taxonomically identified by DNA sequencing of the genes encoding the 16S rRNA subunit (*16S rDNA*), amplified by using the Polymerase Chain Reaction (PCR). The PCR-amplification was performed from total bacterial DNA, which was purified using the InstaGene Matrix resin (Bio-Rad Laboratories Inc., Hercules, California, USA). The resulting mixture (final volume of

50µL), which contained the purified bacterial DNA, NZYTaq II DNA polymerase (MB354) (NZYTech, Lisbon, Portugal) and the amplifying oligonucleotide primers plb16 (5'-AGAGTTTGATCCTGGCTCAG-3') and
mlb16 (5'- GGCTGCTGGCACGTAGTTAG-3') (Sigma-Genosys Ltd., Cambridge, UK) (Kullen *et al.,* 2000).

The sample was subjected to a succession of steps that included: i) initial denaturation (95ºC for 3 min); ii) followed by 35 cycles of denaturation (94ºC for 45 sec); iii) annealing (48ºC for 50 sec); iv) elongation (72ºC for 20 sec); and lastly v) final extension (72ºC for 5 min). All these steps were performed in an Eppendorf Mastercycler thermal cycler (Eppendorf, Hamburg, Germany).

Afterwards, the PCR products were analysed by electrophoresis on 1.5% (w/v) agarose gel (Pronadisa, Madrid, Spain) stained with GelRed (Biotium, California, USA), and finally visualized through the Gel Doc 1000 system (BioRad, Madrid, Spain). The molecular size marker used was HyperLadder 100-bp (Bioline GmbH, Germany). Lastly, the amplicon was purified and cleaned-up through the NucleoSpin Gel and PCR-Clean Up protocol (Macherey & Nagel, Düren, Germany). Later, it was sequenced at the Unidad de Genómica (Parque Científico de Madrid, Facultad de Ciencias Biológicas, UCM, Madrid, Spain). The analysis of the *16S rDNA* target sequence was performed by using the BLAST (Basic Local Alignment Search Tool) program available at the National Centre for Biotechnology Information of the US (NCBI, blast.ncbi.nlm.nih.gov).

During the process, DNA sample from *L. lactis* subsp. *lactis* BB24 was used as positive control and deionized water as a negative control.

3.3. Direct antimicrobial activity test

The target bacterial strain, RBT18, was assayed for direct antimicrobial activity against the four indicator microorganisms mentioned above by a stab-on-agar test (SOAT), a technique previously described by Cintas *et al.,* (1995). Briefly, the selected strain (RBT18) was stabbed onto MRS agar and incubated for 5 h at 30ºC. Subsequently, 15 mL of soft agar (0.8% w/v) medium containing about 1×10^5 cfu/mL of the corresponding indicator strain (previously grown overnight) was added onto the original MRS agar plate, that already contained the grown RBT18 strain. Then, the plate was incubated overnight at 30ºC. This process was repeated for all four pathogen strains, opposing RBT18 against each of them separately.

After incubation, the plates were checked to measure and analyse inhibition zones, which consist of the absence of visible microbial growth around the stabbed cultures.

3.4. Extracellular antimicrobial (bacteriocin) activity

The extracellular antimicrobial activity of cell-free supernatant (CFS) of the selected strain (RBT18) was assayed by an agar well diffusion test (ADT), as described by Cintas *et al.* (1995). The indicator microorganisms used were *L. garvieae* CF00021 and *P. damnosus* CECT4797.

Firstly, multiple supernatants were obtained by centrifugation of the RBT18 culture, at 12,000 rpm at 4ºC for 10 min. Secondly, they were filter-sterilized through 0.22 μm filters (Millipore Corp., Bedford, Massachusetts, USA), and finally stored at -20ºC until further use. Before being used, aliquots of 50 μL of the CFS were submitted to a heat treatment (100ºC, for 10 min). After, they were placed into small wells (5 mm diameter) that were cut in cooled MRS agar (0.8% w/v) plates. These plates were previously seeded separately with each one of the indicator microorganisms (*ca.* 1×10^5 cfu/mL) mentioned above. After 2 h at 4ºC, the plates were incubated at 30ºC for 16 h, allowing the growth of the indicator microorganisms, that were subsequently analysed for the presence of inhibition zones around the supernatant-filled wells.

3.5. PCR-amplification of the extracellular antimicrobial compound (bacteriocin) structural gene

A DNA fragment encoding part of the structural gene of the nisins A/Z was PCR-amplified by using specific primers (NisF: 5'-CTTGGATTTGGTATCTGTTTCG-3'; NisR: 5'-CAATGACAAGTTGCTGTTTTCA-3').

The sample was submitted to a similar protocol than the one stated above: i) initial denaturation (95ºC for 3 min); followed by 35 cycles of ii) denaturation (94ºC for 45 sec); iii) annealing (57ºC for 50 sec); iv) elongation (72ºC for 40 sec); and lastly v) final extension (72ºC for 5 min). All these steps were also performed in an Eppendorf Mastercycler thermal cycler (Eppendorf, Hamburg, Germany).

The corresponding PCR amplified product was subsequently electrophoresed and visualized according to the process described before. The molecular size marker used was the HyperLadder 100-bp (Bioline GmbH, Germany).

The positive control used during this step was *L. lactis* BB24, a NisA producing strain (Cintas, 1995), while two negative controls were also used, a non-nisin-producing bacterial strain (*P. damnosus* CECT4797), and deionized water.

3.6. Bacteriocin cross-immunity assay

CFSs from MRS broth cultures of strain RBT18, *L. lactis* BB24 (NisA) and *L. cremoris* WA2-67 (NisZ) were obtained (Cintas, 1995; Araújo *et al.*, 2015a; 2015b). The supernatants were prepared as described above and submitted to the same heat-treatment (100ºC for 10 min).

This assay intended to test the cross-immunity between the two nisin producing lactococci and the strain RBT18 CFSs through an ADT. Briefly, the three heated CFSs were placed into small wells that were cut in three cooled MRS agar (0.8% w/v) plates (*i.e.* every plate had three wells and each well was filled with a different heated cell-free supernatant). The plates were previously seeded with each one of the three bacterial strains individually. After 2 h at 4ºC, the plates were incubated at 30ºC for 16 h, allowing the growth of the seeded microorganisms, and were subsequently analysed for the presence of inhibition halos around the supernatant-loaded wells.

3.7. Bacteriocin purification

The putative bacteriocin produced by the bacterial strain RBT18 was purified using two different multi-chromatographic procedures. The first protocol used consisted of a modification of the multi-chromatographic procedure described by Cintas *et al.* (1995). The second purification was performed based on the protocol described by Field *et al.* (2012). In both cases, the purifications ended up with a reversed-phase chromatography performed in a Fast Protein Liquid Chromatography system (RP-FPLC) (GE Healthcare, Barcelona, Spain).

3.7.1. Purification procedure I

First a CFS from 1 L RBT18 culture, grown in MRS broth overnight at 30ºC, was obtained through centrifugation (8,000 rpm, 4ºC, 20 min). Then, ammonium sulphate [$(NH_4)_2SO_4$] 50% (w/v) (Merck, Darmstadt, Germany) was added to the supernatant and

the sample was kept with soft stirring at 4ºC for 2 h, followed by a second centrifugation at 8,000 rpm for 30 min. After this, two distinct fractions can be distinguished: AS- and AS+ (as for ammonium sulphate plus), which is a dark brown precipitate. Aliquots of the fractions (AS- and AS+) were heated (95ºC for 10 minutes) and stored at -20 ºC, a process that was performed for every fraction from this one onwards. The fraction AS+ was resuspended in 100 mL of 20 mM sodium phosphate (NaP) buffer (pH 6.0).

This resuspended fraction was subsequently desalted by gel filtration, by using PD-10 columns (GE Healthcare Life Sciences, Barcelona, Spain) (*Figure 3.1*). Firstly, the columns were equilibrated with 20 mM NaP (pH 6.0), then slowly the AS+ fraction was added to the columns obtaining a new fraction: GF+. A new addition of 20 mM NaP (pH 6.0) allowed to obtain the fraction GF-.

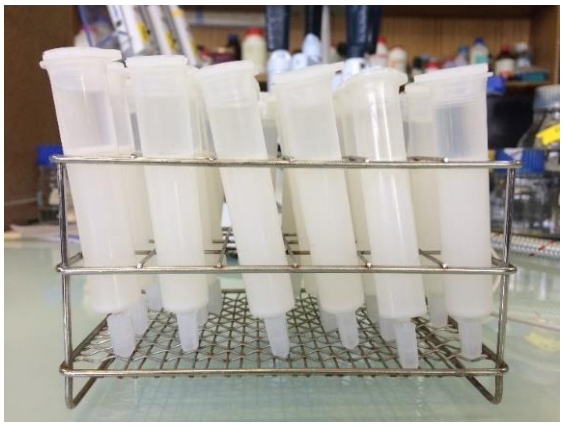

Figure 3.1. PD-10 columns (GE Healthcare Life Sciences) used for gel filtration in the purification procedure I.
Source: Author's own elaboration (2019).

Following gel filtration, was performed a cation-exchange chromatography by using a column containing SP Sepharose Fast Flow resin (GE Healthcare Life Sciences). After equilibration with 20 mM NaP (pH 6.0), the fraction GF+ was slowly added to the column obtaining the fraction SF. The column was then washed again with 20 mM NaP (pH 6.0), resulting in the fraction SW. The final step of the cation exchange was the elution of the retained compounds by using 1 M sodium chlorite (NaCl) in buffer 20 mM NaP (pH 6.0), obtaining the fraction SE, which contained the bacteriocins.

Then a hydrophobic interaction was performed using a smaller column, this time filled with Octyl-Sepharose CL-4B resin (GE Healthcare Life Sciences). The column was equilibrated with $(NH_4)_2SO_4$ in buffer 20 mM NaP (pH 6.0). At the same time, 10% (w/v)

$(NH_4)_2SO_4$ was added to the SE fraction, while softly stirring for 40 minutes. The new mixed SE fraction was added to the equilibrated column, resulting in the fraction OF. The columns was then washed with 10% $(NH_4)_2SO_4$ in buffer 20 mM NaP (pH 6.0), obtaining the fraction OW. Lastly, it was added ethanol (EtOH) (70%) in buffer 20 mM NaP (pH 6.0) to the column, resulting in the final fraction: OE.

3.7.2. Purification procedure II

Initially, the target strain (RBT18) was cultured in GM17 [M17 broth supplemented with 0.5% glucose (v/v)] (Difco, Sparks, Maryland, USA) overnight at 30ºC. Meanwhile, a 1.4 L of TY broth was filtered and passed through a column packed up to one third with Amberlite XAD-16 beads (Sigma Aldrich Co., Ltd., St. Louis, Missouri, USA), where half a litre was retained in the column. To the resulting 900 mL of TY broth was added 50 mL of glucose [at 20% (w/v)], 50 mL of β-glycerophosphate (Sigma Aldrich Co., Ltd.), and it was inoculated with the previously cultured RBT18. The new broth was cultured at 30ºC overnight. The culture was then centrifuged at 7,000 g for 15 min, and the resulting cell pellet was resuspended in 300 mL of 70% isopropanol supplemented with 0.1% trifluoroacetic acid (TFA), and gently stirred at room temperature for 3 h. Meanwhile, the overnight cultured supernatant (SN1 – as it happened during the first protocol, aliquots from every fraction were collected, heated at 95ºC for 10 min and stored at -20ºC) was applied to a smaller column previously packed with 60 g of Amberlite XAD-16 beads and eluted by 70% isopropanol 0.1% TFA into a fraction called AE. After 3 h of stirring, the cell pellet was re-centrifuged at 7,000 g for 15 min again and the new supernatant retained (SN2).

The fractions AE and SN2 were combined (fraction SA), and the isopropanol retained in the solution was evaporated by using a rotary evaporator (Rotavapor R-210, Buchi, Flawil, Switzerland) at 42ºC with an initial pressure of 120 mBar, that was slightly decreased throughout the process, forming the new fraction SAR (*Figure 3.2*). The pH of the fraction SAR was adjusted with sodium hydroxide (NaOH) to pH 4.0 (fraction SAR4).

Figure 3.2. Rotavapor R-210 (Buchi) used in the bacteriocin purification procedure II. Yellow arrow: pressure control. Purple arrow: controller of the sample's rotation. Blue arrow: fraction containing isopropanol and the bacteriocins. Green arrow: evaporated isopropanol. Red arrow: temperature control. *Source: Author's own elaboration (2019).*

The newly adjusted fraction (SAR4) was applied to a 10g (60 mL) Mega BE-C18 column (Agilent, Santa Clara, California, USA), which was assembled to a vacuum pulling system (Model EP-1 Econo Pump, BioRad) (*Figure 3.3*), and pre-equilibrated with methanol and water, obtaining fraction C18F, washed with 30% EtOH (C18W), and finally eluted with 70% isopropanol supplemented with 0.1% TFA (C18E). At this stage, the final volume of C18E was 60 mL. The C18E fraction was filtered through 0.45µm filters (Millipore Corp., Bedford, Massachusetts, USA) and split into six aliquots of 10 mL. The aliquots were separately applied to the Rotavapor R-210 (at 42ºC and 120 mBar) so that the isopropanol evaporated, until they reached a volume of approximately 2 mL each (fractions C18ER).

Figure 3.3. Mega BE-C18 column (Agilent) (yellow arrow) connected to a vacuum pulling system (red arrow) used in purification procedure II.
Source: Author's own elaboration (2019).

3.7.3. Reversed Phase-Fast Protein Liquid Chromatography (RP-FPLC)

In both purifications, the final resulting fractions (OE and CE18ER, respectively) were submitted to a reversed-phase (RP) chromatography column (Source 5RPC ST 4.6/150) (GE Healthcare Life Sciences) in an ÄKTA purifier fast protein liquid chromatography system (RP-FPLC) (GE Healthcare Life Sciences) (*Figures 3.4* and *3.5*). The bacteriocins were eluted from the RP column with a linear gradient of isopropanol in 0.1% (v/v) TFA.

Figure 3.4. RP chromatography column (Source 5RPC ST 4.6/150) (GE Healthcare Life Sciences) used during both purifications.
Source: Author's own elaboration (2019).

Figure 3.5. ÄKTA purifier FPLC system (GE Healthcare Life Sciences).
Source: Author's own elaboration (2019).

The multiple fractions obtained during both purifications were assayed for antimicrobial activity by a microtiter plate assay (MPA) using *L. garvieae* CF00021 as indicator microorganism (*Figure 3.6*). Briefly, two-fold dilutions of the purified fractions were prepared in microtiter plates and the wells were filled up to 200 µL, of which, 50 µL were the purified fraction, and 150 µL were a dilution (in MRS broth) of the fresh overnight cultured indicator microorganism. The microtiter plates were incubated at 30ºC for 16 h, and the growth inhibition of *L. garvieae* CF00021 was assessed spectrophotometrically at 620 nm with a microtiter plate reading system (FLUOstar Optima, BMG Labtech, Ortenberg, Germany) (*Figure 3.7*). Subsequently, the antimicrobial activity was measured in bacteriocin units (BU), which can be defined as the reciprocal of the highest dilution of purified bacteriocin causing 50% growth inhibition (using as control 50% of the turbidity of the wells that did not possess bacteriocins) (Cintas *et al.,* 1995).

The purified fractions demonstrating a high and specific bacteriocin activity were, in both protocols, combined and re-chromatographed through the same RP-FPLC system, until chromatographically pure bacteriocins were achieved.

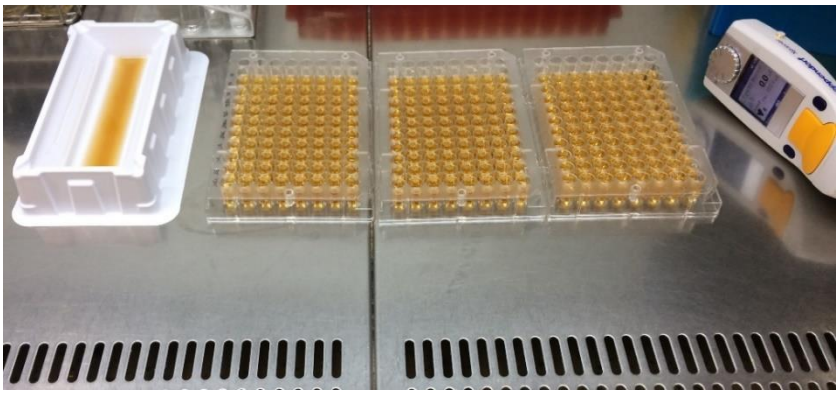

Figure 3.6. Microtiter plates used to assess the antimicrobial activity of the purified fractions against an indicator microorganism (*L. garvieae* CF00021).
Source: Author's own elaboration (2019).

Figure 3.7. Microtiter plate reading system (FLUOstar Optima, BMG Labtech) used during this study.
Source: Author's own elaboration (2019).

3.8. Mass spectrometry analyses (MALDI-TOF/TOF)

The final chromatographically pure peptides were afterwards subjected to mass spectrometry analyses. The process was performed in the Unidad de Proteómica of the Facultad de Farmacia (UCM). In brief, 1 µl of each sample was placed onto a matrix-assisted laser desorption/ionization (MALDI) target plate and allowed to air-dry at room temperature. Then, 0.8 µl of Sinapic acid matrix (Sigma Aldrich Co. Ltd.) in 30% (v/v) acetonitrile 0.3% (v/v) TFA were added and allowed to air-dry at room temperature once again. The MALDI-TOF (Time of Flight) techniques performed used a 4800 Plus Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems, MDS Sciex, Toronto, Canada) equipped with a pulsed nitrogen laser emitting at 337 nm. MALDI-TOF/TOF mass spectra were acquired in a range of mass of 900-6,000 Da with the linear medium mass acquisition method in the positive mode using an accelerating

voltage of 20 kV. Default and plate calibration were performed using the calibration mixture 2 Peptide Mass Standards Kit (Sciex, Framingham, Massachusetts, USA).

3.9. Strain RBT18 Whole Genome Sequencing (WGS)

The bacterial strain RBT18 was successively cultured (in MRS broth at 30ºC overnight) to guarantee a fresh exponential phase culture. Then, some isolated colonies were collected and sent to the Hospital Universitario Ramón y Cajal (Madrid, Spain) in order to perform a WGS assay. The genomic DNA will be isolated and purified by a DNA purification kit from Qiagen and sequenced through a Illumina MiSeq sequencing platform (Illumina Inc., CA, USA). The generated sequences will be assembled using the CLC Genomics Workbench 5.5 (CL Bio., Denmark) program and the coding sequences will be identified and annotated using the RAST program (http://rast. nmpdr.org/). Moroever, the nucleotide sequence will be analysed using several databases, such as Antibiotic Resistance Genes Database (ARB), Comprehensive Antibiotic Resistant database (CARD), Virulence Factor DataBase (VFDB) and Microbial Hazard Gene Information Database. In addition, the genomic sequence of strain RBT18 will be deposited in the international database DDBJ/ENA/GenBank (Bethesda, MD, USA).

4. RESULTS AND DISCUSSION

4.1. Strain RBT18 identification

The BLAST (NCBI) analyses of the *16S rDNA* target sequence revealed a high identity (above 99.79%) with two subspecies of *L. lactis*, namely *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris.* Thus, from now on the strain will be referred as *L. lactis* RBT18 (*Figures 4.1* and *4.2*).

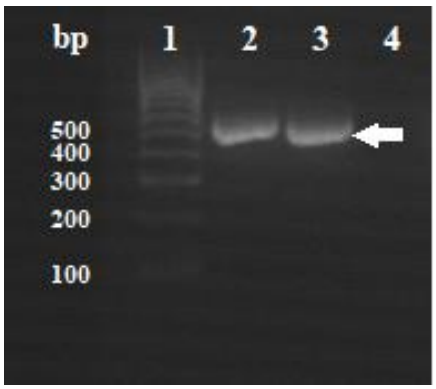

Figure 4.1. Electrophoresis of the PCR amplification.
1) HyperLadder 100-bp (Bioline); 2) *L. lactis* RBT18;
3) *L. lactis* BB24 (white arrow); 4) deionized water
(negative control).
Source: Author's own elaboration (2019).

Nevertheless, both subspecies of *L. lactis* are vastly characterized LAB, associated with having antimicrobial properties and prior aquaculture probiotic application, including several nisin-producing strains (Cintas *et al.*, 1995; Flórez *et al.*, 2008; Zendo *et al.*, 2010; Araújo *et al.*, 2015b; Gómez-Sala *et al.*, 2019).

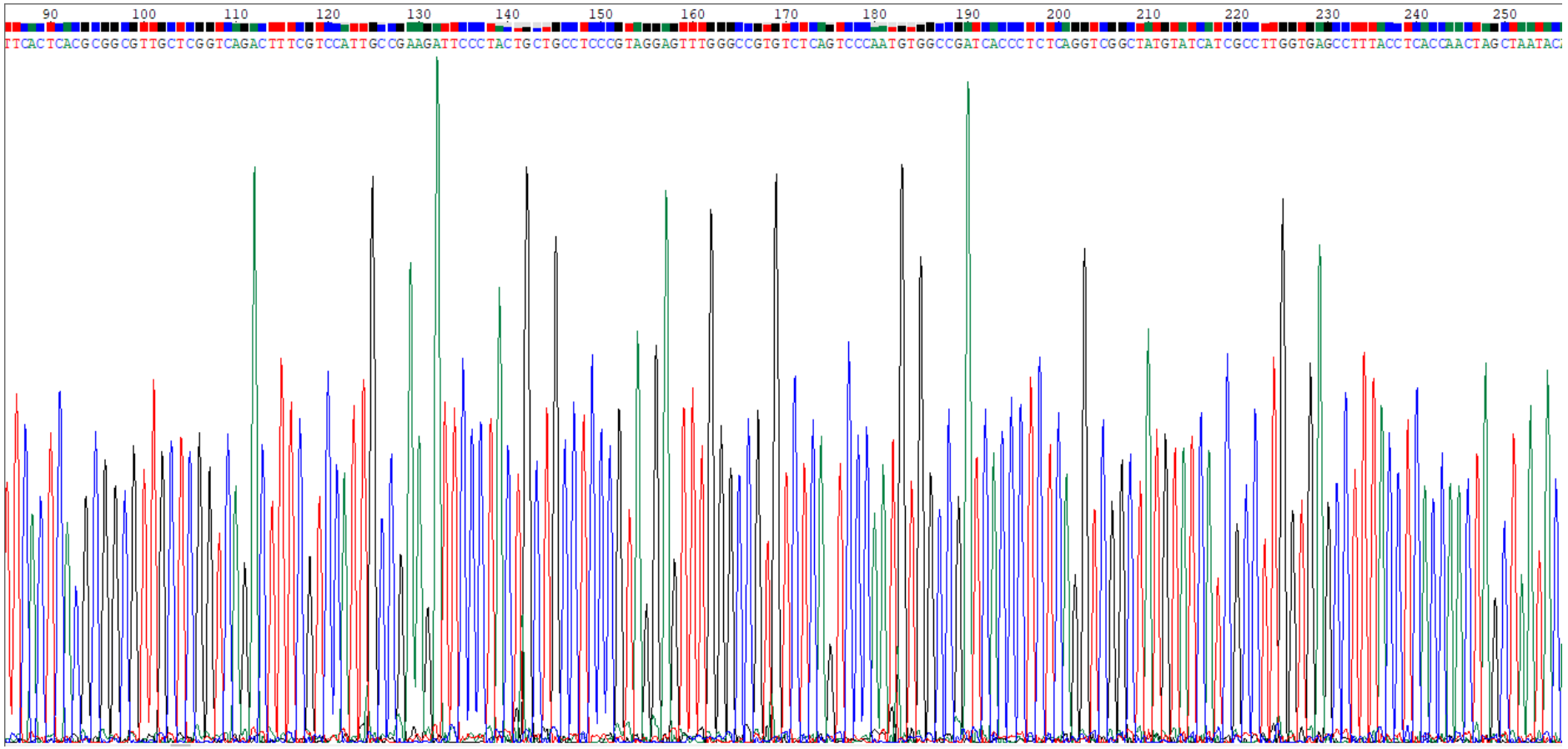

Figure 4.2. Section of the *L. lactis* RBT18 *16S rDNA* sequence (from bp positions 85 to 256) visualized through the program Chromas (Technelysium Pty Ltd., South Brisbane, Australia).
Source: Author's own elaboration (2019).

4.2. Direct antimicrobial activity (SOAT)

L. lactis RBT18 demonstrated through a SOAT a potent direct antimicrobial activity against all four indicator microorganisms (*L. garvieae* CF00021, *L. garvieae* JIP29-99, *L. garvieae* CLG-4 and *P. damnosus* CECT4797) (*Figure 4.3*).

The inhibition halos that were observed were subsequently measured and are registered in *Table IV.1*.

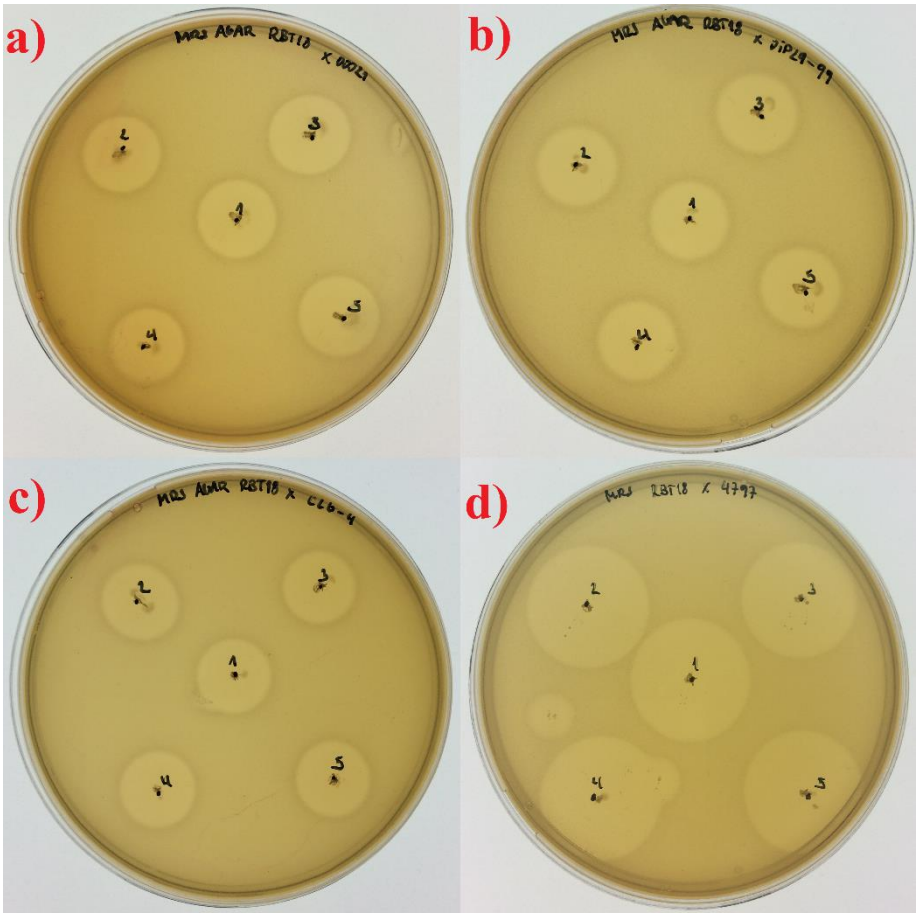

Figure 4.3. Direct antimicrobial activity of *L. lactis* RBT18 against: (a) *L. garvieae* CF00021; (b) *L. garvieae* JIP29-99; (c) *L. garvieae* CLG-4 and (d) *P. damnosus* CECT4797, using a SOAT.
Source: Author's own elaboration (2019).

Table IV.1. Direct antimicrobial activity demonstrated by the strain RBT18, through a SOAT, against four fish pathogens. The results below express the average diameter of the inhibition halos (in mm).

	Indicator microorganisms			
	L. garvieae CF00021	*L. garvieae* JIP29-99	*L. garvieae* CLG-4	*P. damnosus* CECT4797
***L. lactis* RBT18**	16.83	16.44	16.27	28.04

4.3. Extracellular antimicrobial (bacteriocin) activity

L. lactis RBT18 not only exerted direct antimicrobial activity but also extracellular antimicrobial activity by an ADT against the two selected indicator microorganisms: *L. garvieae* CF00021 and *P. damnosus* CECT4797 (*Table IV.2*) (*Figure 4.4*).

The CFSs withstood the heat treatment (100ºC for 10 min) and still remained active, which strongly suggests the involvement of a thermostable compound (*i.e.*., bacteriocins), in their antimicrobial activity (Cintas *et al.*, 2001; Zendo *et al.*, 2010; Gómez-Sala *et al.*, 2019).

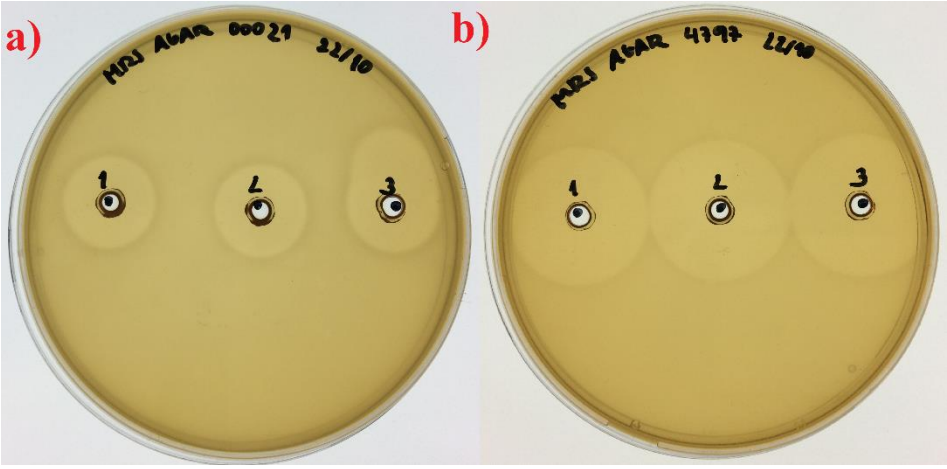

Figure 4.4. Extracellular antimicrobial activity of the CFSs (small carved wells 1, 2, and 3) of *L. lactis* RBT18 against: (a) *L. garvieae* CF00021 and (b) *P. damnosus* CECT4797, by using an ADT.
Source: Author's own elaboration (2019).

Table IV.2. Extracellular antimicrobial activity of cell-free supernatant (through an ADT) from *L. lactis* RBT18 against two indicator microorganisms. The results below express the average diameter of the inhibition halos (in mm).

Cell-free supernatant from *L. lactis* RBT18	**Indicator microorganisms**	
	L. garvieae CF00021	*P. damnosus* CECT4797
	16.52	25.65

4.4. PCR-amplification of the bacteriocin structural gene from L. lactis RBT18

The pair of specific primers for the amplification of NisA/Z's structural gene allowed to amplify, in *L. lactis* RBT18, a fragment of the expected size (132-bp) (*Figure 4.5*).

DNA sample from *L. lactis* BB24, which is a nisin-producing strain (NisA) (Cintas, 1995), was used as a positive control (*Figure 4.5*). After electrophoresis, this amplified fragment appeared to have the same relative length of the target *L. lactis* RBT18 one. Additionally, as expected, no amplified fragments appeared on the negative control lanes.

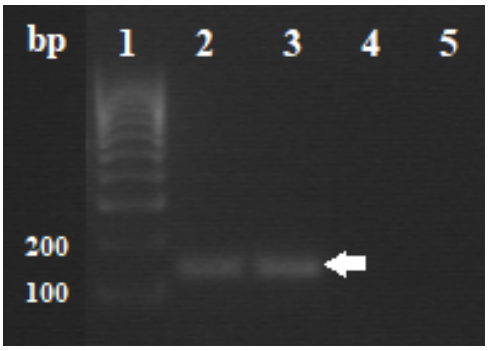

Figure 4.5. Electrophoresis of the amplified fragment of the structural gene of the extracellular antimicrobial compound (bacteriocin) produced by the strain *L. lactis* RBT18. 1) HyperLadder 100-bp (Bioline); 2) cell-free supernatant of *L. lactis* RBT18; 3) cell-free supernatant of *L. lactis* BB24 (white arrow); 4) cell-free supernatant of *P. damnosus* CECT4797; and 5) deionized water.
Source: Author's own elaboration (2019).

Considering that both amplified fragments (CFSs from *L. lactis* RBT18 and *L. lactis* BB24) appear to have similar relative lengths, this indicates that the extracellular antimicrobial compound secreted by *L. lactis* RBT18 is highly similar to the one secreted by the positive control used. Therefore, it is possible to theorize, at this moment, that the extracellular compound secreted by the target LAB could be a variant of nisin, which is the bacteriocin secreted by *L. lactis* BB24 (Cintas, 1995).

4.5. Bacteriocin cross-immunity assays

The cross-immunity assay, performed by an ADT, revealed that none of the three tested CFSs (*L. lactis* RBT18, *L. cremoris* WA2-67 and *L. lactis* BB24) exerted antimicrobial activity against the three respective strains (*Figure 4.6*). Both *L. lactis* BB24 and *L. lactis* WA2-67 are producers of nisin variants, the first producing NisA (Cintas, 1995) and the latter one producing NisZ (Araújo *et al.*, 2015b). Likewise, *L. lactis* RBT18 has been also proven on the previous ADT to exert antimicrobial activity through its heat-treated CFS,

which strongly suggests the involvement of a heat-stable antimicrobial compound, such as a bacteriocin (Cintas *et al.*, 2001; Cotter *et al.*, 2005; Zendo *et al.*, 2010).

As stated previously, nisin producing strains have the ability for self-protection (immunity) against the activity and toxicity of nisin itself (Field *et al.*, 2019). This is achieved by means of at least two different mechanisms, one mediated by a lipoprotein encoded by the gene *nisI*, and the other by the action of an ABC transporter (encoded by the gene *nis*FEG) (Bauer and Dicks, 2005; Wilson-Stanford *et al.*, 2009; Zendo *et al.*, 2010, Field *et al.*, 2019). Taken all together, these results strongly indicate that the antimicrobial activity exerted by *L. lactis* RBT18 might be due to the production of the bacteriocin NisA/Z.

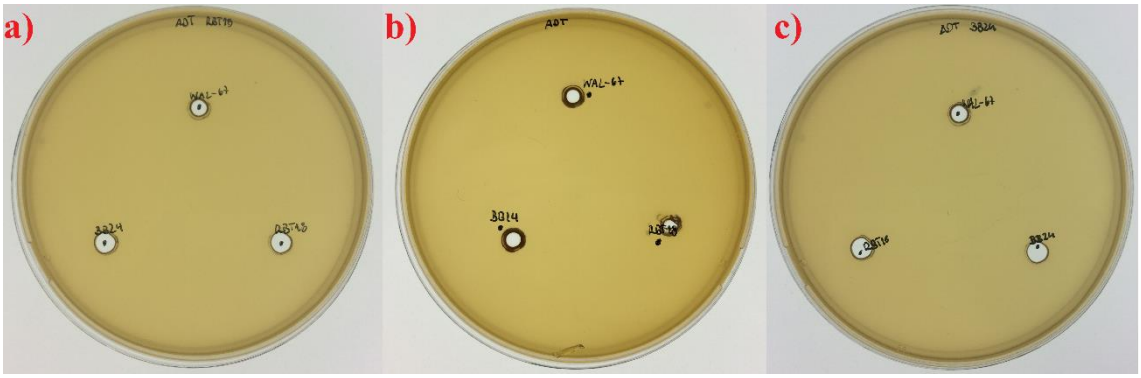

Figure 4.6. Extracellular antimicrobial activity of *L. lactis* RBT18, *L. cremoris* WA2-67 and *L. lactis* BB24, against the three same strains, by using an ADT. The assay opposed the cell-free supernatants of *L. lactis* RBT18, *L. cremoris* WA2-67 and *L. lactis* BB24 (each small carved well contained one cell-free supernatant of each strain *per* plate) against the same seeded microorganisms: (a) *L. lactis* RBT18, (b) *L. cremoris* WA2-6, and (c) *L. lactis* BB24.
Source: Author's own elaboration (2019).

4.6. Bacteriocin purification and mass spectrometry analyses

4.6.1. Purification procedure I

The last RP-FPLC performed at the end of the first purification protocol resulted in a highly distinctive absorbance peak. This absorbance peak reflects the elution of the bacteriocin secreted by *L. lactis* RBT18, which demonstrated antimicrobial activity towards the indicator microorganism used (*L. garvieae* CF00021). Additionally, the remain fractions' activity results, measured by MPA, are summarized in *Table IV.3.*

The absorbance peak eluted at approximately 58% (v/v) isopropanol (elution buffer) in aqueous 0.1% (w/v) TFA. The fraction corresponding to the absorbance peak represented a yield of approximately 77% and a 342,000-fold increase in specific antimicrobial activity (*Table IV.3*). Additionally, the peak's purity and molecular mass were assessed by MALDI-TOF/TOF mass spectrometry. MALDI-TOF/TOF mass spectrometry analysis allowed the identification of a peptide with a molecular mass of 3,332.17 Da, which fits with that of NisZ (3,330 Da), and a second peptide with a molecular mass of 3,348.97 Da (*Figure 4.7*). Therefore, suggesting the presence of a NisZ oxidized form (16 Da added), likely derived from the oxidation of a lanthionine ring. In this respect, not only is NisZ the most common natural variant of NisA, but also several *L. lactis* strains have been previously associated with the production of this same variant (Mulders *et al.*, 1991; Guder *et al.*, 2000; Park *et al.*, 2003; Araújo *et al.*, 2015b).

Table IV.3. Final RP-FPLC of bacteriocin from *L. lactis* RBT18 using the multi-chromatographic purification procedure I.

Purification stage	Volume (mL)	Total A_{280}[a]	Total activity (10^3 BU)[b]	Specific activity[c]	Increase in specific activity[d]	Yield (%)[e]
Culture supernatant	1,000.0	14,036.0000	1,280.00	91.19	1.00	100.00
Ammonium sulphate precipitation	100.0	2,055.4000	8,192.00	3,985.60	43.70	640.00
Gel filtration chromatography	172.0	1,075.3400	880.64	818.93	8.98	68.80
Cation-exchange chromatography	50.0	152.1000	750.00	4,930.96	54.07	58.59
Hydrophobic-interaction chromatography	15.0	17.7900	614.40	34,536.26	378.71	48.00
Reversed-phase chromatography	1.5	0.0315	983.04	31,207,619.00	342,211.00	76.80

[a]Absorbance at 280 nm (A_{280}) multiplied by the volume (mL). [b]Antimicrobial activity in bacteriocin units per millilitre (BU/mL) and multiplied by the total volume (mL). [c]Specific antimicrobial activity expressed as the total antimicrobial activity (BU) divided by total A_{280}. [d]Specific antimicrobial activity of a fraction (BU/A_{280}) divided by the specific antimicrobial activity of the supernatant (BU/A_{280}). [e]Yield expressed as the total antimicrobial activity of a fraction (BU) multiplied by 100 and divided by the total antimicrobial activity of the supernatant (BU)

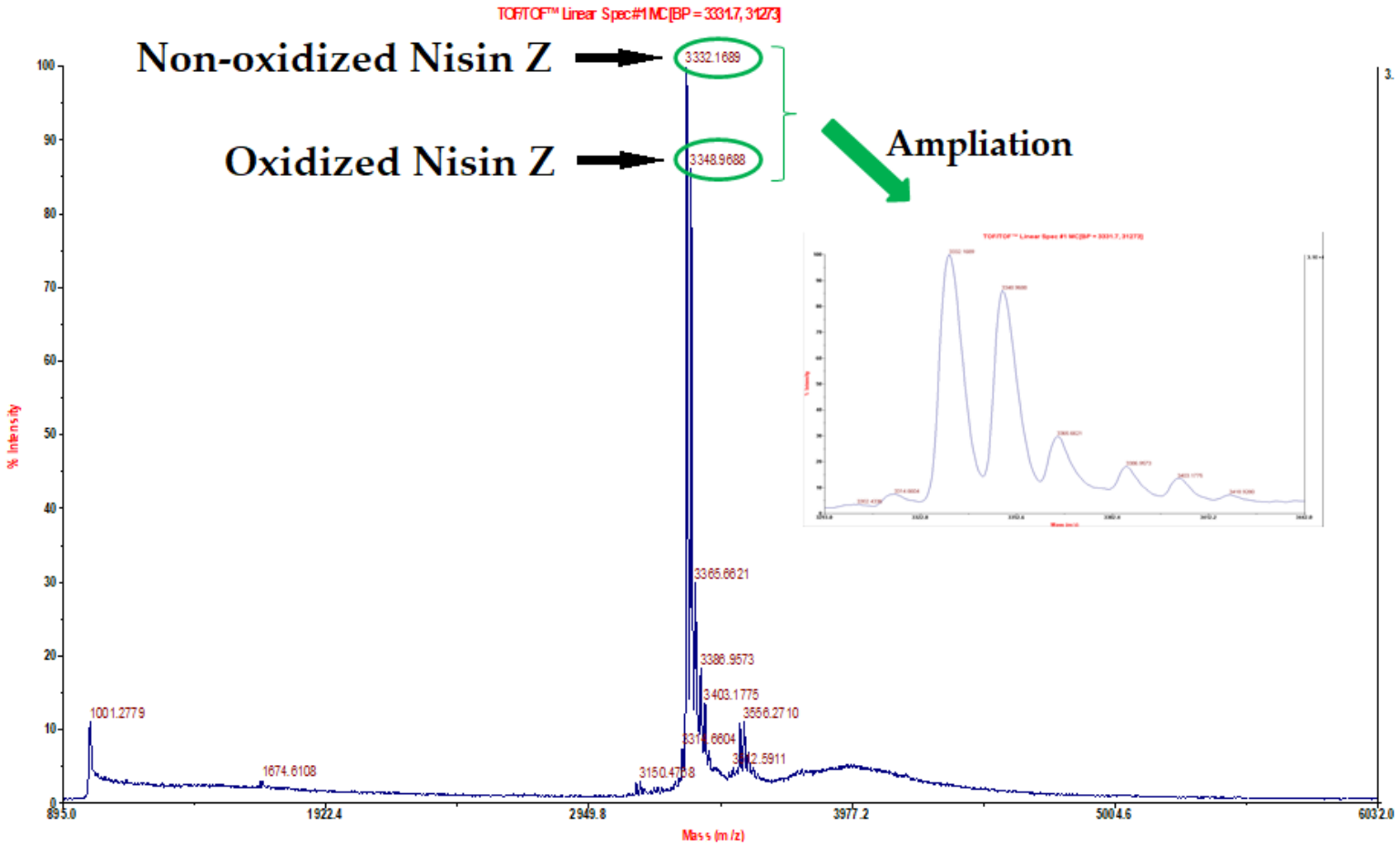

Figure 4.7. MALDI-TOF/TOF mass spectrometry analysis of NisZ from *L. lactis* RBT18 purified after the final RP-FPLC using the multi-chromatographic purification procedure I.
Source: Unidad de Proteómica, Facultad de Farmacia UCM (2019).

4.6.2. Purification protocol II

The last RP-FPLC performed in sequence of the second purification procedure displayed two easily distinctive absorbance peaks. These elution peaks suggested the presence of the heat-stable antimicrobial compound (*i.e.*, bacteriocin) secreted by *L. lactis* RBT18, which demonstrated activity towards the indicator microorganism used (*L. garvieae* CF00021). Additionally, the fractions successively collected throughout the protocol exerted activity towards *L. garvieae* CF00021 and their activity results are summarized in *Table IV.4*. Both elution peaks surged slightly earlier when compared with the peak derived from the purification procedure I. While the first activity peak eluted at approximately 55%, the second one eluted at nearly 58% (v/v) isopropanol in aqueous 0.1% (w/v) TFA (*Figure 4.8*). These fractions (corresponding to the first and second elution peaks) represented approximately a yield of 0.4 and 3.8%, and a 2,700- and 35,000-fold increase in specific antimicrobial activity, respectively (*Table IV.4*).

The two main peaks had their purity and molecular mass separately assessed by MALDI-TOF/TOF mass spectrometry. The second elution peak revealed a peptide with a molecular mass of approximately 3,330.09 Da (*Figure 4.9*), being, as in the previous purification procedure, highly identical to the reported molecular mass of NisZ (3,330 Da) (Guder *et al.*, 2000). Similar to the RP-FPLC performed after the first purification procedure, the MALDI-TOF/TOF analysis revealed an additional peptide with a molecular mass of approximately 3,346.26 Da, which once again suggests the presence of an oxidized form of NisZ (16 Da more) (*Figure 4.10*).

Table IV.4. Final RP-FPLC of bacteriocin from *L. lactis* RBT18 using the multi-chromatographic purification procedure II.

Purification stage	Volume (mL)	Total A_{280}[a]	Total activity (10^3 BU)[b]	Specific activity[c]	Increase in specific activity[d]	Yield (%)[e]
First culture supernatant	1,000.0	7,082.0000	2,560.00	361.47	1.00	100.00
Fraction AE	250.0	238.0000	1,280.00	5,378.15	14.88	50.00
Second culture supernatant	150.0	120.9000	768.00	6,352.35	17.57	30.00
Fraction SA	400.0	337.6000	2,048.00	6,066.35	16.78	80.00
Fraction SAR4	125.0	93.7500	640.00	6,826.67	18.88	25.00
Fraction C18E[f]	60.0	28.5000	2,457.60	86,231.58	238.55	96.00
Fraction C18ER[f]	2.0	1.2500	327.68	262,144.00	725.20	12.80
First elution peak (oxidized NisZ)	0.5	0.0105	10.24	975,238.10	2,697.90	0.40
Second elution peak (non-oxidized NisZ)	0.6	0.0078	98.30	12,603,077.00	34,865.23	3.84

[a]Absorbance at 280 nm (A_{280}) multiplied by the volume (mL). [b]Antimicrobial activity in bacteriocin units per millilitre (BU/mL), and multiplied by the total volume (mL). [c]Specific antimicrobial activity expressed as the total antimicrobial activity (BU) divided by total A_{280}. [d]Specific antimicrobial activity of a fraction (BU/A_{280}) divided by the specific antimicrobial activity of the first supernatant (BU/A_{280}). [e]Yield expressed as the total antimicrobial activity of a fraction (BU) multiplied by 100 and divided by the total antimicrobial activity of the first supernatant (BU). [f]The fraction C18E was separated into six fractions of 10 mL each, consequently each fraction went under an evaporation process until they reached an approximated volume of 2 mL each, constituting separately the fractions C18ER.

Figure 4.8. Final RP-FPLC of the multi-chromatographic purification procedure II used to purify NisZ from *L. lactis* RBT18.
Source: Author's own elaboration (2019).

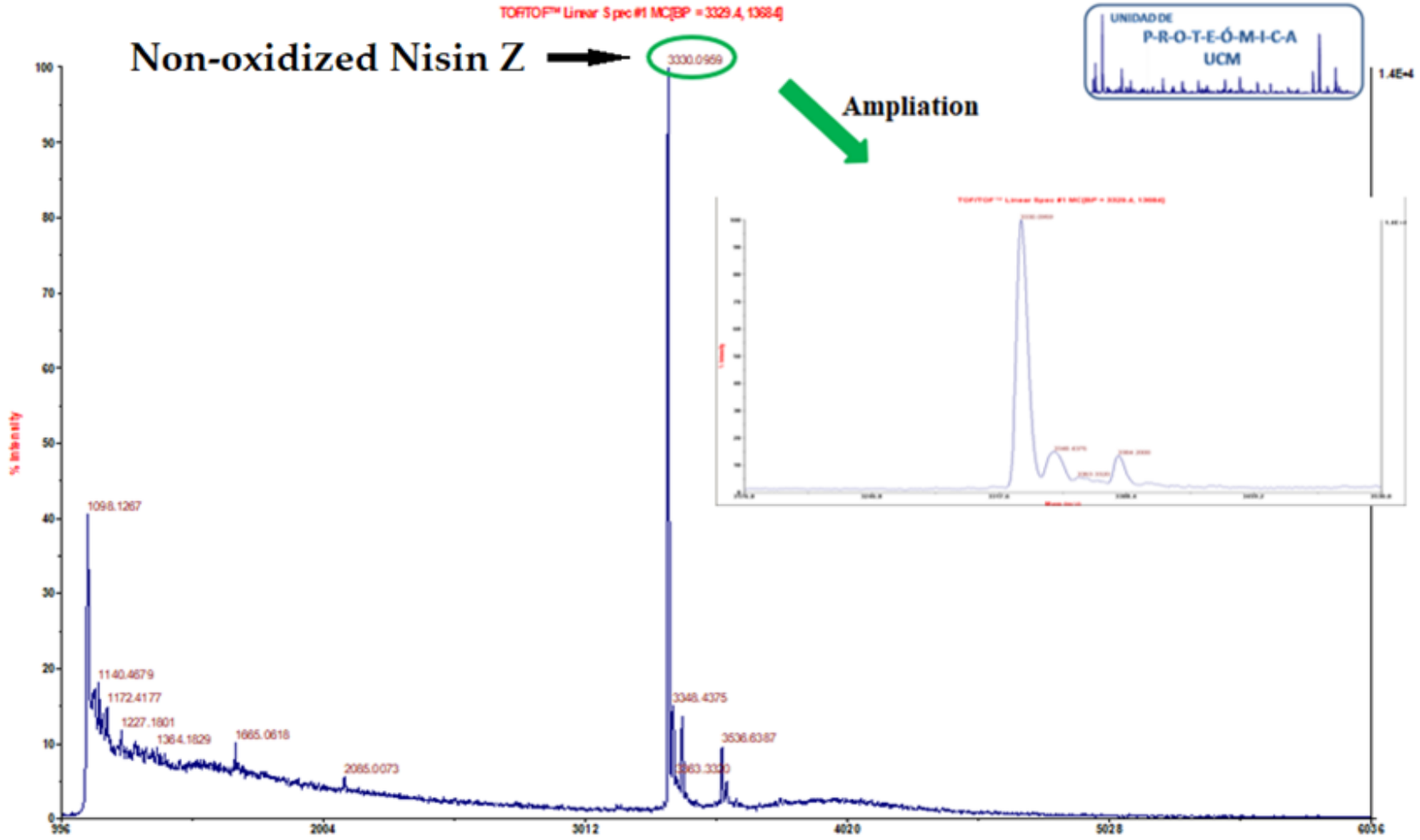

Figure 4.9. MALDI-TOF/TOF mass spectrometry analysis of the non-oxidized form of NisZ from *L. lactis* RBT18 purified after the final RP-FPLC using the multi-chromatographic purification procedure II.
Source: Unidad de Proteómica, Facultad de Farmacia UCM (2019).

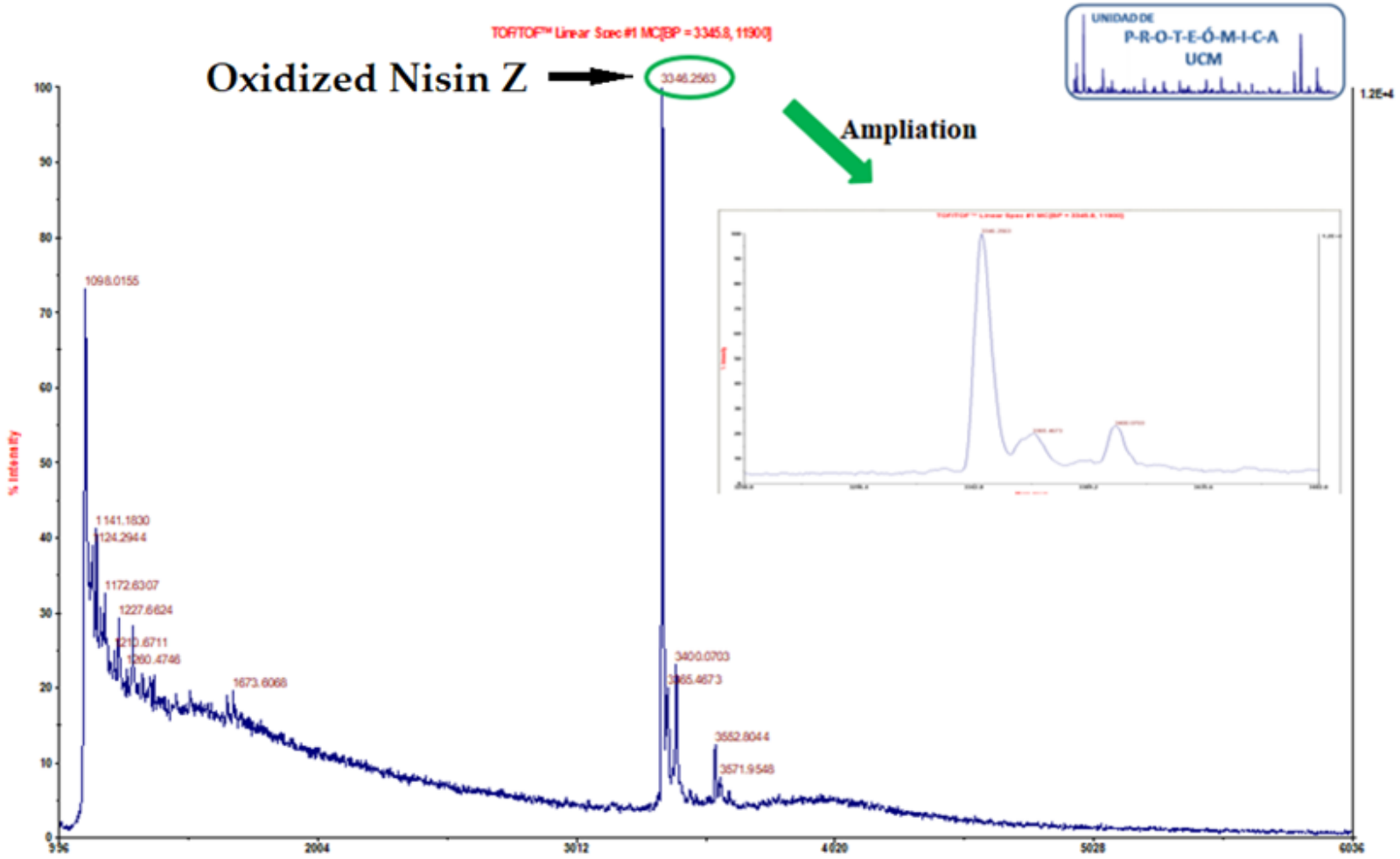

Figure 4.10. MALDI-TOF/TOF mass spectrometry analysis of the oxidized form of NisZ from *L. lactis* RBT18 purified after the final RP-FPLC using the multi-chromatographic purification procedure II.
Source: Unidad de Proteómica, Facultad de Farmacia UCM (2019).

Table IV.5. Comparison between the final RP-FPLC of bacteriocins from *L. lactis* RBT18 of the two multi-chromatographic purification procedures.

	Volume (ml)	Total A_{280}[a]	Total activity (10^3 BU)[b]	Specific activity[c]	Increase in specific activity[d]	Yield (%)[e]
Procedure I						
Reversed-phase chromatography	1.5	0.0315	983.04	31,207,619.00	342,211.00	76.80
Procedure II						
Oxidized NisZ	0.5	0.0105	10.24	975,238.10	2,697.90	0.40
Non-oxidized NisZ	0.6	0.0078	98.30	12,603,077.00	34,865.23	3.84
Oxidized NisZ + Non-oxidized NisZ	1.1	0.0183	108.54	5,931,148.00	16,408.41	4.24

[a]Absorbance at 280 nm (A280) multiplied by the volume (ml). [b]Antimicrobial activity in bacteriocin units per milliliter (BU/ml) and multiplied by the total volume (ml). [c]Specific antimicrobial activity expressed as the total antimicrobial activity (BU) divided by total A280. [d]Specific antimicrobial activity of a fraction (BU/A280) divided by the specific antimicrobial activity of the first supernatant (BU/A280). [e]Yield expressed as the total antimicrobial activity (BU) of a fraction multiplied by 100 and divided by the total antimicrobial activity (BU) of the CFS.

During this second purification procedure, the oxidized form of NisZ demonstrated a decrease (estimated in 12-times) in the specific antimicrobial activity, if compared to the non-oxidized form of NisZ (second elution peak) (*Table IV.5*). It is likely that this decrease might be related to the nisin's mechanism of action. In this respect, the interactions between nisin and the peptidoglycan precursor named lipid II (lipid II-mediated mechanisms), not only allow the formation of highly specific pores, but also interfere with the bacterial cell wall synthesis. Together, they constitute part of the hypothesised mechanisms by which nisin variants exert their bactericidal activity on target bacteria (Bauer and Dicks, 2005; Zendo *et al.*, 2010). As described by Wilson-Stanford *et al.* (2009), it is theorized that this observed decrease in the antimicrobial activity of the oxidized form of NisZ could be due to its lack of ability to properly bind with and sequester the lipid II molecules. Ultimately, it is this lack of binding ability that leads to a significant decrease in the bioactivity of nisin. Hence, this bioactivity decrease of the oxidized form of NisZ could increase the chances of bacterial pathogens to evade and resist the antimicrobial activity of nisin. Moreover, it is also possible that a nisin resistance could play a role in the resistance to other antimicrobial substances, increasing the concern and interest to develop methods to reduce and counteract nisin's oxidation (Wilson-Stanford *et al.,* 2009; Field *et al.*, 2019).

Additionally, when comparing both procedures, the first purification procedure demonstrated, to a certain extent, a higher effectiveness. This higher effectiveness regards variables such as: i) increase in specific activity, and ii) antimicrobial activity yield, which both directly impact the purpose of this work, the purification of NisZ. In this respect, the first purification procedure has an outcome of an increase in the specific activity and antimicrobial activity yield 21 and 18-times higher respectively, than using the second purification procedure (*Table IV.5*).

4.7. L. lactis RBT18 Whole Genome Sequencing (WGS)

To date, and partially due to the SARS-CoV-2 pandemic that impacted the world, the results of the genome sequencing assay performed at the Hospital Universitario Ramón y Cajal (Madrid, Spain) are still not available, and therefore, cannot be presented in this memory.

The functional analysis of the complete genome sequence or the draft genome sequence of *L. lactis* RBT18 will allow the identification of potential virulence genes, genes that encode resistances to antibiotics and genes that encode the synthesis of bacteriocins, peptides or proteins related to its probiotic potential. In addition, the determination of the genomic sequence of the *L. lactis* RBT18 will facilitate its recognition by the EFSA for evaluation of their potential as probiotic in aquaculture and probably other biotechnological applications, such as the production of compounds to be used as additives in food and feed.

5. CONCLUSIONS

Firstly, *L. lactis* RBT18, isolated from cultured rainbow trout (*Oncorhynchus mykiss*, Walbaum), exerts a strong direct and extracellular antimicrobial activity against *L. garvieae* and other ichthyopathogens, being this antimicrobial activity heat-resistant (100ºC, 10 min), and thus suggesting the involvement of a thermostable antimicrobial compound (*i.e.*, bacteriocin). Moreover, the procedures carried out in this work, such as the PCR-analysis, the direct and extracellular antimicrobial assays, and the cross-immunity assay, demonstrated to be a suitable strategy for the preliminary identification of lactococcal nisin-producing strains. Additionally, the two multi-chromatographic purification procedures performed were suitable for the purification of NisZ, both in its native and oxidized forms. In this respect, the first purification procedure demonstrated to be more appropriate and effective for the purpose, with an increase in specific activity and a higher antimicrobial activity yield 21-18-fold higher than using the purification procedure II.

Finally, the observable diminished antimicrobial activity exerted by the oxidized form of NisZ rises concerns about the evasion and resistance of bacterial pathogens to the nisin's bioactivity. Thus, it is necessary a better understanding of the bacteriocin oxidation process, as well as its implications on pharmacodynamics, pharmacokinetics and development of pathogen resistances.

Therefore, further experiments are required to assess the *in vitro* and *in vivo* safety and efficiency of the bacteriocinogenic strain *L. lactis* RBT18 as a probiotic for aquaculture, but also to optimize the environmental conditions required to reduce nisin oxidation, and consequently the rising of bacterial pathogen resistances.

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