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BIOTECHNOLOGY FOR A SUSTAINABLE AQUACULTURE: SELECTION OF SAFE MARINE BACTERIA PRODUCERS OF LC-PUFAs AND ANTIMICROBIALS

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GENERAL ABSTRACT

The increasing awareness among consumers of the health benefits associated with fish intakes has led to the increase in fish consumption. Thus, the challenge for aquaculture industry is to meet demand of fish. This would be achieved through a sustainable growth and expansion of the aquaculture systems where a maximal reduction of wastes and an optimized fish production are targeted. Indeed, the fish processing industries produce high amounts of by-products that can be exploited to obtain products with potential applications in different industrial sectors like feed, food or cosmetics. On the other hand, optimization of aquaculture fish production could be supported by the application of eco-friendly antimicrobial compounds and by the research of alternative sources of long-chain polyunsaturated fatty acids (LC-PUFAs).

Marine environment constitutes one of the most promising resources for the discovery of new microbial species with the ability to produce compounds that could be of interest for a sustainable aquaculture industry, including antimicrobial compounds and LC-PUFAs. Marine microorganisms are in fact able to survive in extreme and competitive habitat by producing non-ribosomal secondary metabolites, bacteriocins, lysozymes, proteases, surfactants/biosurfactants and exopolysaccharides (Prieto *et al.*, 2012; Baharum *et al.*, 2010; Schinke *et al.*, 2017). Antimicrobial compounds produced by marine microorganisms (like for example bacteriocins) have attracted a considerable interest to researchers due to their potential as eco-friendly solution to reduce the use of antibiotics (associated with antibiotic resistance) and vaccines (present excessive cost and not available for all fish species) for the control of bacterial diseases in aquaculture.

LC-PUFAs, in particular in EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid) compounds are very important ingredients in fish feed because they are involved in reproduction and larvae development. Moreover, high levels of LC-PUFAs in fishes may be a source of these essential nutrients known to have a proven role in human health and which have to be supplied by the diet. Currently fish oil is used as PUFAs source for fish feed, but it is not enough to meet the demand of the growing aquaculture industry; furthermore the composition and quantity of PUFAs in fish oil depend on the fish species, season and geographical location of the capture. Microorganisms isolated from marine environment a sustainable source of PUFA allowing a stable supply of these compounds.

To date, several studies were carried out on the valorisation of fishing industry by-products/wastes and many of the products obtained are available in commerce (Zhang *et al.*, 2019). However, only very few reports are available on the selection/application of probiotics in aquaculture as well as on the use of antimicrobial compounds from microbial origin for the control of bacterial pathogens. In this context, the aim of the research activity is to select novel safe marine bacteria able to produce LC-PUFAs and/or antimicrobial compounds active against aquaculture fish pathogenic bacteria with the perspective of their application in aquaculture.

The first part of the work has been dedicated to the selection of LC-PUFAs bacterial producers. For this purpose, a collection of 209 marine bacterial isolates obtained from water and sediment samples recovered in the Mediterranean Sea was screened in order to select new eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) bacterial producers. Among them, 95 bacterial isolates are potentially LC-PUFAs producers based on colourimetric screening. These 95 isolates were dereplicated by ITS typing resulting in the selection of 48 haplotypes. Among them, 31 isolates quickly growing were selected for further studies. Using GC-FID analysis, the detection of LC-PUFAs was confirmed from 15 isolates belonging to the genera *Marinobacter*, *Halomonas* and *Thalassospira*. Among them, one isolate named *Marinobacter* sp. G16.20 was found to be a potentially high LC-PUFA producer; producing relatively high levels of DHA in particular. The maximum achieved productivity of DHA by this isolate was 1.85±0.371 mg/g, representing 45.89%of the total fatty acids detected under the analyses conditions applied in this study.

Although there are reports on the production of LC-PUFAs from marine bacteria, to the best of our knowledge, there have been no reports on the investigation of these interesting biotechnological traits from the genera reported in this study. Moreover, this is the first report on the production of LC-PUFAs from Mediterranean Sea.

In the second part of the work, efforts has been concentrated on the selection of antimicrobial compounds producers and the characterization of the stability of the produced compounds. For this purpose, the 15 isolates having the ability to produce DHA together with other 26 marine isolates from collection were considered. The isolates were grown in modified mineral salt medium broth (mMSM). After bacterial growth, the cell free supernatant (CFS) was recovered by and used to evaluate antimicrobial activity by agar well-diffusion method. As indicator strains, four aquaculture fish pathogenic bacterial species including *Yersinia ruckeri*, *Vibrio harveyi*, *Photobacterium damselae* and *Flavobacterium psychrophilum* were used. Among these 41 marine isolates screened, none inhibited the

growth of *F. psychrophilum* while 35, 17 and 4 exhibited antibacterial activity towards *Y. ruckeri*, *P. damselae* and *V. harveyi*, respectively. Interestingly, 4 isolates were producers of one or more molecules active against all three fish pathogens and 13 inhibited the growth of both *Y. ruckeri and P. damselae.* For most of these 17 isolates, growth and production of antimicrobial compounds were not correlated. To evaluate the stability of the antimicrobial activity, CFS were subjected to pH adjustments (pH 4- pH 10), thermic (60 °C; 80 °C and 120 °C) and proteolytic treatments: before being used for the evaluation of the antibacterial activity and the results were expressed as residual antibacterial activity (RA %). The results showed that 8 marine isolates were able to produce antimicrobial compounds that exhibited 100% residual activity after exposure to pH/temperature extremes and after proteinase K treatment. These results provide interesting inputs for further studies dedicated to the recovery of these active compounds and for the elucidation of their nature. In the present study, a collection of marine bacteria was screened for the selection of isolates which have the ability to produce antimicrobial compounds, potentially bacteriocins, active against four fish pathogens responsible for diseases in aquaculture. Identifying marine isolates that produce molecules with antibacterial activity, allows controlling the pathogens of fish and representing a potential eco-friendly solution to reduce the massive use of conventional antibiotics and antimicrobial agents that have negative consequences on environment and human health (Sahoo *et al.*, 2016; Rather *et al.*, 2017; Scott *et al.*, 2018).

In conclusion, among a large collection of marine bacterial isolates screened, 10 strains able to produce both antimicrobial compounds active against different aquaculture fish pathogenic bacteria and LC-PUFAs were selected. These results are interesting and promising since these isolates belong to bacterial species that have never been reported to be involved in human or animal pathology and exhibited traits that give them high potential for the application in aquaculture in order to optimize fish production.

1. Biotechnology for the sustainability of the aquaculture systems: state of the art and future perspectives

1.1 Abstract

In the last years, global aquaculture production has been growing (FAO, 2018) and the request of fish feed as well as the quantities of waste and by-products are increasing accordingly. Aquaculture industry is searching solutions to improve the economic, environmental and social sustainability through the development of measures for improving farmed fish quality and safety, reducing waste and valorising by-products.

The high stocking density increases fish stress and make them susceptible to diseases; probiotics and prebiotics may be added to fish feed as eco-friendly alternative to overcome antibiotics treatment drawbacks. Several studies report that probiotics have a direct growth-stimulation effect on fish and improve water quality in tanks. Moreover, probiotics isolates from marine environment were found to be able to produce polyunsaturated fatty acids (PUFAs) and consequently enhance the accumulation of these compounds in fish.

Fish by-products can be used for extraction of several medium-high value products (i.e. bioactive peptides, antioxidant compounds), as substrate for microbial biopolymers production and also as an alternative source for biodiesel production.

Recent review papers on the sustainable aquaculture reporting only the current aquaculture scenario (Dauda *et al.*, 2019; Sicuro, 2019), the use of eco-friendly treatments for fish dieses (Dawood *et al*., 2019; Lieke *et al.*, 2019; Chauhan *et al.*, 2019) or the production/recovery of bioactive compounds from fish waste (Marti-Quijal *et al.*, 2020; Abuine *et al.*, 2019) but without providing a complete overview on all of these.

In this review we provide a complete overview on strategies for improving aquaculture sustainability including the use of probiotics and prebiotics and the potentialities of high-added value products obtainable from fish by-products.

Keywords**:** *aquaculture sustainability, fish probiotic, fish prebiotic, fish by-products valorisation.*

1.2 Introduction

The contribution of aquaculture to global food production in the fishing industry is expanding, reaching 46.8% in 2016 compared to 25.7% in 2000. The world aquaculture production of food fish in 2016 was 80 million tonnes (FAO, 2018).

The growing demand of fish and the need to minimize environmental impact urge aquaculture industry to develop sustainable processes aimed at: i) improving quantity and quality of fish, ii) containing costs, iii) reducing environmental threats by minimizing wastes and valorizing by-products (Jennings *et al.*, 2016; EU, 2019). Optimization of fish production/quality/safety could be supported by the application of eco-friendly alternative to classic antimicrobial compounds, such as probiotics and prebiotics. The ecosustainability of the aquaculture systems could be achieved through the exploitation of the high amounts of by-products produced by the fish processing industries and which are rich in proteins and fats that can be treated to obtain bio-based materials with potential applications in different industrial sectors like feed, food or cosmetics (Akhter *et al.*, 2015; Sprague *et al.*, 2016; Sicuro, 2019).

In this chapter, an overview of the current scenario on the application of probiotics/prebiotics in aquaculture as well as on the processes developed for the valorization of fish processing by-products/wastes is provided. Furthermore, the challenges to overcome for the development of an eco-sustainable aquaculture system through biotechnology exploitation are discussed.

1.3 Probiotic and prebiotics: eco-friendly agents for diseases control in aquaculture

The spread of diseases is one of the critical limiting factors in the aquaculture system. Indeed, the high stocking density increases fish stress and reduces water quality, both of which optimal for fish pathogens. Most of the disease-causing microorganisms in marine fish are opportunistic pathogens generally present as part of normal seawater microflora able to cause diseases in the host when it is under stressed conditions (Lazado *et al.*, 2015). The most common diseases among fish include vibriosis, yersiniosis and fish tuberculosis (Table 1.1) (Rather *et al.*, 2017).

Four strategies are widely used for containing the effects of fish pathogenic microorganisms, namely disinfectants for water (i.e. hydrogen peroxide and peracetic acid), antibiotics, vaccines and medicinal plant products (Lieke *et al.*, 2019). Disinfectants for water allow temporary results, therefore the treatments must be repeated several times. Antibiotics, in addition to eliminating bacterial pathogens, had negative consequences on fish and human health by inducing well known antibiotic-resistance phenomena (Alonso *et al.*, 2019). Currently, vaccination is the most important disease prevention and control method in aquaculture, especially in salmonid aquaculture where it has reduced disease outbreaks and limited the addition of antibiotics (Munang'andu, 2018). Vaccines consist of antigens derived from inactivated pathogens (by heat or other means) and adjuvants; this formulation stimulates fish immune response and induce a specific long-term protection against a specific disease. To date, 19 major companies market fish vaccines, for example against *Yersinia ruckeri*, *Vibrio* spp. and *Photobacterium damselae subspecies piscicida*, but not all of them are commercially available worldwide (Table 1.1). However, vaccines based strategies are not cost effective. Compared with terrestrial animals, fish need a large antigen dose: complex manufacturing procedures for antigen production (i.e. are requires specific artificial medium and controlled operating conditions) make the production process complex and expensive. Some species are too vulnerable to handle the stress induced during the vaccination or may develop severe side effects post vaccination and most importantly, this could not be a solution for all fish species since some do not develop a reactive immune system (Henriksson *et al.*, 2018). Moreover, vaccines are not available for all fish pathogens and in all the countries (Table 1.1). These disadvantages limit the growth of aquaculture vaccines market (Adams, 2019; Sommerset *et al.*, 2005).

Table 1.1 Major fish pathogenic bacteria and related diseases.

Fish pathogen bacteria	Disease	Fish species affected	Vaccine
Vibrio spp. (*Vibrio alginolyticus, V. anguillarum*)	Vibriosis	All fish species (major: salmonids, cod, sea bass)	Commercially available (globally)
Photobacterium damselae, Photobacterium damselae subspecies piscicida	Ulcer Disease, Pausterellosis	All fish species (major: sea bass, sea bream, amberjack)	Commercially available in Mediterranean, not in Japan
Yersinia ruckeri	Enteric red mouth (ERM)/Yersiniosis	Salmonids, rainbow trout	Commercially available in Europe, Chile, Canada/USA
Flavobacterium columnare	Columnaris disease or Saddleback disease	Salmonids, catfish and many other fish	Commercially available in Chile and USA
Flavobacterium psychrophilum	Flavobacteriosis	Salmonids	Commercially available in Chile, Canada/USA (West)
Flavobacterium psychrophilum	Rainbow trout fly syndrome	Salmonids	Not commercially available
Lactococcus garvieae	Lactococcosis	All fish species (major: raibow trout, amberjack)	Commercially available in Italy, France, UK and Japan
Streptococcus iniae, *Streptococcus phocae*	Streptococcosis	Tilapia, hybrid striped bass, rainbow trout	Commercially available Asia, not in Chile

Recently proposed strategies aimed at limiting the spread of "antibiotic-resistance" phenomenon and avoiding the drawbacks of vaccination, trying to formulate an adequate diet for aquaculture fish without negative effects for fish and human health (Santos and Ramos, 2018). The key factors of such approach are: i) the prevention of any significant alteration of aquaculture and of the environment (neither additives supplementation nor any treatment are needed); ii) the large share of individuals targeted; iii) the limited influence of individual physiologic peculiarities on the overall effectiveness; iv) the reasonable cost of the process. Among such methods to improve aquaculture profitability and sustainability, the stimulation of immune response of fish through the administration of probiotics and prebiotics is the most promising.

1.3.1 Use of probiotics in aquaculture

In recent years much attention has been played on the use of eco-friendly feed additives for disease control and prevention, such as probiotics (Jahangiri and Esteban, 2018).

FAO and World Health Organization Working (WHO) defined probiotics as "*living microorganisms which, once administered in appropriate amounts, confer a health profit on the host*" (FAO/WHO, 2001). These microorganisms (non-pathogenic) colonize and proliferate in the gut of host (fish); there they exert the effect by competing with fish pathogens, by stimulating the immune system, and by releasing metabolites with antimicrobial properties (e.i. bacteriocins, siderophores and lactic acid). Several studies suggest that the employment of probiotics is also associated with an increase growth, digestion, stress tolerance, reproductive capacity of fish and provide fatty acids and vitamins (Nayak, 2010; Lazado *et al.*, 2015).

Most probiotics have been isolated from microbiota of aquatic organisms. For example, the *Lactobacillus* and *Bifidobacterium* species present in the gut of freshwater fish, produce lactic acid that improve fish growth, nutrient digestibility of feed and immune system response (Alonso et al, 2019; Kuebutornye *et al.*, 2019).

The general procedures to select probiotics include: 1) collect most-detailed scientific data available; 2) purchase potential probiotic strains (non-pathogenic); 3) test the ability of potential probiotic to produce, in pure culture, antimicrobial compounds active against fish pathogen strain; 4) test the effect of probiotic (positive at step 3) on fish (host); 5) selection and validation of appropriate method for probiotic administration; 6) economic cost/benefit analysis.

Council Directive 70/524/EEC report the list of microorganisms authorized as feed probiotics in European Union; the list includes *Bacillus cereus var. toyoi*, *Bacillus licheniformis*, *Bacillus subtilis*, *Enterococcus faecium*, *Lactobacillus casei*, *Lactobacillus farciminis*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Pediococcus acidilactici*, *Saccharomyces cerevisiae* and *Streptococcus infantarius*. In addition, other probiotics are commercialized on the market, but their use has not officially approved by the European Commission (Pandiyan *et al.*, 2013). Antony *et al.* (2011) have reported as a mixture of *Bacillus* sp. and *Vibrio* sp. positively affects the growth and survival of white shrimp and presents a protective effect against *V. harveyi*; this is due to the stimulation of the immune system. Nikoskelainen *et al.* (2003) reported that the administration of *Lactobacillus rhamnosus* in an amount of 10^5 CFU/g feed stimulates the respiratory burst in rainbow trout.

1.3.1.1 Methods of administration of probiotics in fish

Immunomodulatory activity of probiotics depends on various factor like method of administration, dose of probiotics and the duration of administration is very important to certify their success.

The probiotics can be administered as dietary supplements, by injection, via microencapsulation or by direct addition to water column.

Dietary supplements (via live food or pellet food) consist of the integration of probiotic to the feed (in form of spores or freeze-dried cultures) that, when administered for consecutive days, guarantee a successful colonization of gastrointestinal tract (GUT). Although this method is easy, the viability should be checked in feed pellets (Kumar *et al.*, 2016). Alternatively, probiotics can be injected (intraperitoneal): this method is the most efficient but it takes a long time and it is laborious (Verschuere *et al.*, 2000). In microencapsulation/bioencapsultaion, microbial cells at high density are encapsulated/immobilized in colloidal matrix (e.g. alginate, carboxymethylcellulose, pectin or chitosan) providing physic and chemical protection, facilitating the release of the probiotics in the intestine, avoiding the exposure to digestive juices and consequent inactivation/hydrolysis/degradation (Shefat, 2018). Microcapsule can be enriched by various kinds of nutrients for improve the nutritional status of fish (Kumar *et al.*, 2016). Direct addition of probiotic to water column is faster and more effective in marine environments, because of higher probiotic uptake by fish on account of intensive drinking activity in these environments.

1.3.1.2 Mechanism of action of probiotics

Although over the last decades many microorganisms have been tested for use as probiotics in aquaculture system with promising results (Kuebutornye *et al.*, 2019; Chauhan *et al.*, 2019), the exact mechanism of interaction has not been completely clarified, the positive effects include: inhibition of pathogenic bacteria, antiviral effects, enhancement of the immune response against infectious pathogens, growth promotion and improvement of water quality.

Inhibition of pathogenic bacteria

Probiotic confer resistance to diseases by competitive inhibition of pathogen bacteria, production of inhibitory compounds and alteration of enzymatic activities of pathogens.

Probiotic generated compounds include hydrogen peroxides, organic acids (that lower the environments pH of GUT), bacteriocins and bacteriocin-like inhibitory substances that prevent proliferation of opportunist pathogen and even eliminate these.

Antiviral effects

Although the exact mechanism by which some probiotics exerts its antiviral effects is not known, laboratory tests indicates that the inactivation of viruses can occur by chemical and biological substances, such extracellular agents of bacteria (Pandiyan *et al.*, 2013); hence probiotics are supposed to contribute to that effect.

Enhancement of the immune response against infectious pathogens

Some probiotics strengthening innate immune responses by increasing the activation of macrophages, the production of antibodies, T-cell proliferation and production of interferon (Tuan *et al.*, 2013); Michael *et al.* (2014) also reported that fish larvae shrimps and other invertebrates have immune systems not completely mature for their response to infection. Same studies have demoted that probiotic administration improve and stimulate the non-specific defence (Kumar *et al.*, 2016).

Growth promotion

Probiotics have a direct growth-promoting effects of fish ascribed to their enzymatic activities. Enzymes produced by probiotics improve nutrient availability, digestibility and utilization of nutrients. Studies reported that same microorganisms such *Agrobacterium* sp., *Pseudomonas* sp., *Brevibacterium* sp., *Microbacterium* sp. and *Staphylococcus* sp. may contribute to nutritional process in Artic charr (*Salvelinus alpinus* L.), participate in the digestion process by producing extracellular enzymes such as proteases, lipases and providing necessary growth factors (Ringø *et al.*, 1995; Kumar *et al.*, 2016). Wanka *et al.* (2018) reported that the probiotic administration is associated with an increase of essential polyunsaturated fatty acids (PUFAs) and their precursors. PUFAs are important in fish reproduction and larvae development. Eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA) are the principal components of bacterial membrane lipids of various marine bacteria and are very important for human health because they contribute to optimal development of the nervous system, reduce cardiovascular risk and exhibit anti-inflammatory effects. Consequently, probiotics formulation consisting of a mixture of

marine bacteria isolated from deep sea would easily establish in host gut (Antony *et al.*, 2011).

Improvement of water quality

High concentration compounds such ammonia, nitrate and nitrite cause mass mortality in aquaculture system. As reported by Brailo *et al.* (2019), denitrifying bacteria having the capacity to carry out ammonia and nitrate oxidation might be employed to overcome such issue. For example, *Bacillus subtilis* has been widely administered as a probiotic agent to control the water quality, resulting in significantly reduced ammonia levels and fish mortality (Jahangiri and Esteban, 2018).

1.3.2 Use of prebiotic in aquaculture

The prebiotic concept was first introduced by Gilbson and Roberfroid in 1995 (Gibson and Roberfroid, 1995) and subsequently updated. They defined a prebiotic as *"indigestible food ingredients that selectively and beneficially affects the host by stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health*" (Gibson *et al.*, 2004). Prebiotics, unlike probiotic, are not microorganism and have limited influence in natural environment (Yousefian and Amiri, 2009). Nonetheless, fish prebiotics exert similar effects, such as the increase of growth rate, the improvement of immune system (Song *et al.*, 2014), the shaping of gut microbiota, the prevention of pathogens colonisation such as *Salmonella, Listeria* and *Escherichia coli* (Ringø *et al.*, 2010), the production of systemic effect which favour the utilization of feed ingredient and neutralize toxins. There are many prebiotic used as immunostimulants in aquaculture, but the main are fructooligosaccharides (FOS), galactooligosaccharides (GOS), inulin, mannoligosaccharides (MOS), some proteins, peptides and lipids.

The properties of prebiotics include resistance to gastric acidity, susceptibility to digestive enzymes, induced hydrolysis and gastrointestinal adsorption and selective stimulation of the growth associated with health; furthermore, they must be fermented by non-pathogenic intestinal microflora as that would in turn enhance the positive effects exherted by probiotics. Fermentation of prebiotics by gut microbiota produces Short-Chain Fatty Acids (SCFAs), including lactic acid, butyric acid, and propionic acid (Davani-Davari *et al.*, 2019), as well as PUFAs. These compounds can have multiple effects in aquaculture systems. Zhao and

colleagues (2019) have reported that dietary PUFAs level influences endogen PUFAs composition and improves fish growth (Tu *et al.*, 2010). The degree of PUFAs in fish (as calculated on the total fatty acids), as well as mammalian and bacteria cells plays a pivotal role in determining membrane fluidity, hence providing the correct adaptation to swinging environmental temperatures (Yoshida *et al.*, 2016); in addition, they are precursors for prostaglandins (PGs) and leukotrienes production (Oliva-Teles, 2012). Moreover, US Food and Drug Administration (FDA) reported that the consumption of up to 3 g/d of marine-based PUFAs (in people attaining to a diet rich in fatty fishes) is important for preventing and controlling cardiovascular diseases, neurological disorders, inflammatory state and metabolic disorders (Melo *et al.*, 2019; Kaviani *et al.*, 2019; Parolini, 2019). Therefore, an increased degree of PUFA in fishes would a relevant influence on human health. Fish consumption is in fact recommended in the 2015–2020 Dietary Guidelines for Americans and by the American Heart Association (Rimm *et al.*, 2018).

Although most studies on prebiotic supplementation indicate some beneficial effects, possible negative effects should not be disregarded and must be further elucidated.

Only recently studies started to unravel the mechanisms of action of prebiotic on fish. However, those studies are still in their early stages and much work still needs to be performed to provide information on age and size responses, timing and duration of prebiotic administration. To validate prebiotic effects, it is important to include studies in environmental stressful conditions such as temperature, hypoxia, salinity or fish density (Guerreiro *et al.*, 2018).

1.4 Fish by-products valorisation

In recent years, a growing awareness of the benefits associated with fish consumption has led to a rapid development of the aquaculture sector. Aquaculture system, produces 32 million tonnes of waste from the production system, including unused biomass (Pędziwiatr *et al.*, 2017; Dauda *et al.*, 2019).

In 2013, based on the goals of sustainable aquaculture and fishing sectors, the European Union Common Fisheries Policy (CFP) are pursued two objectives: i) a drastic reduction/ban of discards; ii) to make the best possible use of captured resource or biomass (Jenning *et al.*, 2016; EU, 2019). As consequence, in these years the worldwide researchers have been focus their research for fish by-products valorisation process in order to obtain/recover new medium-high added value products (i.e. gelatin, collagen, chitosan, biodiesel and active peptides) (Marti-Quijal *et al.*, 2020). Currently, products obtained from fish protein

hydrolysates are commercially available in US, Canada, UK, Japan, North America and Italy. For example, Seacure® is prepared by deep-ocean white fish and is used as supplements for digestive health; Amizate® is prepared by Atlantic salmon and have effects on muscular and circulatory system; Stabilium 200® is prepared by Garum armoricum and is used as support for memory and cognitive system (Chalamaiah *et al.*, 2012)

Further results of some studies showed that fish by-products valorisation processes and traditional waste management systems presented similar economic impacts; however, a significant benefit can be achieved through valorisation of fish by-products (Lopes *et al.*, 2015). Moreover, in order to improve the environmental and economic sustainability of fish by-products valorisation process, Vázquez and colleagues (2019) have also developed a set of sequential and complementary steps in order to recover oils, gelatins, antioxidants compounds and fish peptone; these last compound was used as low cost source of organic nitrogen for microbial antimicrobial compounds production.

Medium-high added value marine products

The aquaculture waste generated include carcass, heads, viscera, tails, skin, vertebral column, fins, scales and its composition is heterogeneous in according to species, sex, fish size, season, rearing techniques, husbandry and fishing area (Villamil *et al.*, 2017; Pędziwiatr *et al.*, 2017; Dauda *et al.*, 2019; de Medeiros *et al.*, 2019). However, most of the fish waste contain proteins (15-30%), fats (0-25%), elements (i.e. calcium 5.8%, phosphorous 2.04% and sodium 0.61%) and moisture (50-80%): these components can be use as raw material to recovery/produce medium-high value products (Ghaly *et al.*, 2013) (Table 1.2).

In the next section, we will discuss the principal medium-high value by-products obtainable from fish waste.

Table 1.2 Principal medium-high value products obtainable from fish byproducts/wastes.

Medium-high added value products	Fish waste	Application	Reference
Collagen and gelatin	Skin	Medical and pharmaceutical industries (e.i. drugs and genes delivery); Food industry; Cosmetic industry (treatment for nail and hair)	Kim and Mendis, 2006; Ghaly *et al.*, 2013; Villamil *et al.*, 2017; Zhang *et al.*, 2019.
Bioactive peptides (antioxidant, antihypertensive, antithrombolitic, immunomodulatory, anticoagulant, antimicrobial agents)	Muscle, Viscera, fish saurce	Food industry; Medical industries; Nutraceutical products.	Marti-Quijal *et al.*, 2020; Najafian and Babji, 2012.
Fish oil	Skin, head, frame and gut	Human consummation	de Medeiros *et al.*, 2019
Chitin and chitosan	Shells and shellfish wastes	Food, agricultural, biotechnological and pharmaceutical industries	Nisticò, 2017 Kim and Mendis, 2006
Fertilizer	Fish waste and effluent	Agricultural use	Radziemska *et al.*, 2019
Biodiesel	Skin	Biofuel	da Costa Cardoso *et al.*, 2019 Al Azad *et al.*, 2019 Saifuddin and Boyce, 2017
Microbial growth media	Viscera, stomach, various fish	Biological and biotechnological a laboratory	Vieira *et al.*, 2005 Villamil *et al.*, 2017
Proteolytic enzymes	Internal organs	Food, nutraceutical and detergent industry	Ghaly *et al.*, 2013

Collagen and gelatin

The fish skin waste is rich in collagen and gelatin. These are two different forms of same macromolecule: gelatin derived by partial hydrolysis of collagen. Fish collagen and gelatin have unique characteristic: i) are rich in non-polar amino acid (such as glycine, alanine, valine and proline); ii) are non-mammalians essential nutrients, thus not banned by habits of some religious creeds which would otherwise induce nutrition deficiencies; iii) remove the risk of mad cow disease or bovine spongiform encephalopathy (BSE).

Collagen are generally extracted with acid treatment followed by thermal treatment for conversion to gelatin state. Gelatin peptides, extracted enzymatically from fish skin, have antioxidative and antihypertensive properties can be associated with their unique repeating sequence of amino acid (glycine-proline-alanine). Collagen is used in medical and pharmaceutical industries as drugs carrier, gene delivery agents and as support for tissues

and organs formation. Gelatin is incorporated into food such as desserts (Kim and Mendis, 2006; Villamil *et al.*, 2017; Huang *et al.*, 2019; Zhang *et al.*, 2019)

Bioactive peptides

Recent studies are focused on the research of bioactive peptides from fermentation processes (Saadaoui *et al.*, 2019). Bioactive peptides consist of short amino acids chains (from 2 to 20) that are inactive within the original protein (precursor) but show specific functions after release by hydrolysis. Several enzymes from microorganisms are used to obtain biologically active peptides: these activities depending on their amino acid sequences, molecular weights and type of raw material (muscle, viscera or fish sources). After enzymatic digestion, bioactive peptides are recovered with ultrafiltration membrane system based on their molecular weights.

Bioactive peptides isolated from various fish by-products have shown antihypertensive, antioxidant, antithrombolitic, immunomodulatory, anticoagulant, antiplatelet properties. Antioxidant compounds have been obtained used the fermentation with lactic acid bacteria and same fungal species of the genera *Aspergillus* (Marti-Quijal *et al.*, 2020); the antioxidant activity of the bioactive peptides hydrolysates was measured by the ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) and DPPH (2,2-diphenyl-1-picrylhydrazyl) assay (Najafian and Babji, 2012). Recently, several studies have demonstrated that that fish peptides are capable of accelerating calcium absorption: these peptides could be used in the treatment of osteoporosis and Paget's disease (Kim and Mendis, 2006).

A wide variety of fish produce and exude antimicrobial peptides as a primary innate immune strategy. In medical applications, antimicrobial peptides are sometimes preferred to conventional bactericidal antibiotics since they kill bacteria faster and do not induce antibiotic-resistance (Shahidi and Zhong, 2008). Antimicrobial activity of hydrolysates or peptides have been tested by agar diffusion assay. Researchers have reported that almost all fish antimicrobial peptides (e.i. Hepcidin TH1–5 (Naqash and Nazeer, 2011) and Efap (Salampessy *et al.*, 2010) have antibacterial or bacteriostatic functions against several Gram-negative and Gram-positive strains; other peptides can be used as antiviral and antifungal agents (Rajanbabu *et al.*, 2011).

Fish oil

The fat content of fish ranges between 2 and 20% depending on species, dietary, environmental, geographic area, reproductive and seasonal variations. A significant portion of total fatty acids is represented by PUFAs, eicosapentanoic acid (EPA) and docosahexaenoic acid (DHA) being the most abundant. As previously reported these class of compounds are important in human health to fight against numerous diseases. Many fish raw would be a potential source to extract fish oil for human consummation. High-speed centrifugation, low temperature solvent extraction and supercritical fluid extraction are utilized to extract fish oil (Kim and Mendis, 2006).

Biodiesel

The standard fuels used presently is petroleum diesel: these is non-renewable source and emit toxic elements into environment. Solutions to word's energy and environmental crisis must be found in the use of low-cost raw materials for biodiesel production, for instance. Biodiesel is a renewable energy source and emit less polluting gases (da Costa Cardoso *et al.*, 2019). The crude fish oil extracted from fish waste represent a good source for biodiesel production because is rich in lipids, stable, low cost and it specific calorific value is similar to that of petroleum oil (Lopes *et al.*, 2015; Al Azad *et al.*, 2019; Moon *et al.*, 2009; Vázquez *et al.*, 2019). Biodiesel is obtained from fish oil using a short-chain alcohol (like methanol or ethanol) in the presence of a chemical or biochemical catalyst (acid, base or enzyme) (Ghaly *et al.*, 2013): when the transesterification reaction occurs, triacylglycerols (TAG) will be transformed into fatty acid alkyl ester (FAME) (Arumugam and Ponnusami, 2017). The biodiesel production process is linked to alcohol: oil molar ratio, catalyst, reaction time and temperature (da Costa Cardoso, 2019).

Microbial grown media

Increasing demand for microbial growth media for the biotechnological fermentation industry has raised attention for new and inexpensive peptone sources because the nitrogen source made the most expensive part of microbial growth media. Several studies have shown that due to its favourable amino acid balance and high protein content, fish materials represent a potential source of industrial peptones (Fallah *et al.*, 2015). Fish peptones obtained from enzymatic hydrolysis of fish tissues (like viscera and stomachs) were tested as growth medium for different pathogens and probiotics microorganisms that are interest to aquaculture (Vieira *et al.*, 2005; Villamil *et al.*, 2017). Chen *et al.* (1997) have assessed Poly-

β-hydroxybutyrate (PHB) production in *Azotobacter vinelandii* UWD in presence of fish peptone; as reported by Page et al (1992) in presence of fish peptones the cells grown is very slowly but the metabolism remain active for PHB accumulation.

Unfortunately, the standardization of fish raw material is a critical point because its composition different from one batch to another, thus their hydrolysis is not comparable (Villamil *et al.*, 2017).

Enzymes

A range of enzymes is available in internal organs of fish. Fish enzymes including pepsin, trypsin, chymotrypsin, collagenase, elastase, chitinase and chitosanase; that compared to their homologues from other organisms, exhibit high catalytic efficiency at low temperatures and concentrations, stability over a broad pH range. These characteristics have made them suitable for different applications in food, nutraceutical and detergent industry. When the fish enzymes do not have direct application in the field of functional foods and nutraceutical industry, they can be utilized to produce bioactive components (Andevari *et al.*, 2019).

Chitin and chitosan

Chitinases and chitosanases isolated from internal organs of fish can be used to recover chitin and chitosan from shells and shellfish wastes. Shrimp shell contain a huge amount of chitin (8-10%) which is an expensive ingredient used in many foods, cosmetics and pharmaceutical products. Chitin, chitosan and their oligomers are nontoxic, biocompatible and biodegradable materials that have important structural and functional proprieties useful in food, agricultural, biotechnological and pharmaceutical industries. As identified by many researchers, chitosan and its oligomers are effective in reducing LDL-cholesterol level in liver and blood (Kanauchi *et al.*, 1995), possess antitumor activities (Jeon and Kim, 2002), stimulate fibroblast production, antioxidant and immunostimulant properties (Kim and Mendis, 2006).

Fertilizer

Composting initiatives using fish by-product have been carried out in various regions in search of alternative and viable techniques for transforming fish waste into useful agricultural products. Fish waste and effluent is sustainable for agricultural due to its richness in nutrients such as nitrogen, phosphorus and calcium. Several fertilizers made of

fishmeal are now commercially available and have been authorized for use in organic agriculture (Radziemska *et al.*, 2019).

1.5 Conclusion and future perspectives

The consumption of fish is highly recommended in human diet and a number of crucial nutrients, or precursors can be obtained from fishes. Thus, aquaculture sector is the fastest growing food sector. Aquaculture industry is searching solutions to improve the economic, environmental and social sustainability through the development of measures for improving farmed fish quality and safety, reducing waste and valorising by-products. In order to preserve the functionality of fish/aquatic animals cultivation, probiotics and prebiotics are attracting large interest.

Probiotics are eco-friendly alternative to antibiotics and chemicals in aquaculture. These probiotics, as well as the prebiotics, seem to be able to enhance the growth and survival rates; this is due to the enhancement of the immune response against infectious pathogens and improvement of water quality.

However, the results of administration of probiotics and prebiotics depend on age and species fish, pathogen or parasite, method of administration, dosage and temperature. Moreover, concerns have been raised about the viability of probiotics in supplementing product, persistence in the gut, horizontal gene transfer from pathogenic bacteria and enhanced inflammatory responses (Lieke *et al.*, 2019).

Future studies of transcriptome and proteome profiling of gut microbiota could clarify the mechanisms of action of probiotics and prebiotics and allow to study molecular methods to improve their effectiveness (Guerreiro *et al.*, 2018). To validate probiotic and prebiotic effects, it is important to clarify the relationship between the efficacy of probiotic and prebiotics administration methods (Jahangiri and Esteban, 2018).

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2. Aim of the work

The increasing of global aquaculture production (FAO, 2018) required measures to improve the economic, social and environmental sustainability of this sector, especially in terms of fish quality/quantity and waste reduction/valorization.

Optimization of fish production could be supported by probiotics and prebiotics administration (Akhter *et al.*, 2015).

Probiotics are living microorganisms which, once administered in appropriate amounts, confer a health profit on the host (fish) (FAO/WHO, 2001). These microorganisms (non-pathogenic) proliferate in the gut of fish competing with fish pathogens by releasing metabolites with antimicrobial properties (e.i. bacteriocins), increase growth, digestion, stress tolerance and reproductive capacity of fish (Nayak, 2010; Lazado *et al.*, 2015).

LC-PUFA (long chain polyunsaturated fatty acids) are important components in fish feed because are involved in reproduction and larvae development. Moreover, high levels of LC-PUFAs in fish may be a source of these compounds for humans with positive effect in health status. In facts, these compounds have important effects in human health because reduce the risk of atherosclerosis, improve blood pressure, prevent blood clots, have anti-inflammatory, anti-depressant and anti-cancer properties. Currently the major source of PUFAs is fish oil but for the sustainability of aquaculture sector is important research alternative sources of these compounds. Cells membranes of marine microorganisms are rich in PUFAs because are important for low temperature adaptation: these microorganisms could be used as alternative and competitive PUFAs source.

To date, several studies were carried out on the valorisation of fishing industry by-products/wastes and many of the products obtained are available in commerce (Zhang *et al.*, 2019). However, only very few reports are available on the selection/application of probiotics in aquaculture as well as on the use of antimicrobial compounds from microbial origin for the control of bacterial pathogens. In this context, the aim of the research activity is to select novel safe marine bacteria able to produce LC-PUFAs (Chapter 3) and/or antimicrobial compounds active against aquaculture fish pathogenic bacteria (Chapter 4) with the perspective of their application in aquaculture.

3. Med Sea marine bacteria as a potential source of long chain polyunsaturated fatty acids

3.1 Abstract

A collection of 209 marine bacterial isolates obtained from water and sediment samples recovered in the Mediterranean Sea was screened in order to select new long-chain polyunsaturated fatty acids (LC-PUFAs), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), bacterial producers. Among them, 95 bacterial isolates are potentially LC-PUFAs producers based on colourimetric screening. These 95 isolates were dereplicated by ITS typing resulting in the selection of 48 haplotypes. Among them, 31 isolates quickly growing were selected for further studies. Using GC-FID analysis, the detection of LC-PUFAs was confirmed from 15 isolates belonging to the genera *Marinobacter, Halomonas and Thalassospira*. Among them, one isolate named *Marinobacter* sp. G16.20 was found to be a potentially high LC-PUFA producer; producing relatively high levels of DHA in particular. The maximum achieved productivity of DHA by this isolate was 1.85±0.371 mg/g, representing 45.89% of the total fatty acids detected. Although there are reports on the production of LC-PUFAs from marine bacteria, to the best of our knowledge, there have been no reports on the investigation of these interesting biotechnological traits from the genera reported in this study. Moreover, this is the first report on the production of LC-PUFAs from strains obtained from Mediterranean Sea.

***Keywords*:** Mediterranean Sea; Long-chain polyunsaturated fatty acids, LC-PUFAs, *Marinobacter* sp.; Docosahexaenoic acid, DHA; Eicosapentaenoic acid, EPA.

3.2 Introduction

Marine environment constitutes one of the most promising resources for the discovery of new microbial species with the ability to produce compounds that could be of interest for industrial biotechnology. It has been reported that both microbial diversity as well as metabolic capabilities of marine-dwelling microorganisms are still underexplored. Hence studies directed to unravelling the microbial diversity and discover new metabolic capabilities are very encouraged for a sustainable blue growth. Specifically, marine microbes and their products including enzymes, antimicrobial compounds as well as pigments and long-chain polyunsaturated fatty acids (LC-PUFAs) are getting increasing interest in the frame of the marine biotechnology sector (Ryan *et al.*, 2010; Abd El Razak *et al.*, 2014). LC-PUFAs are essential nutrients known to have a proven role in human health which have to be supplied by the diet (Calder, 2015; Tocher, 2015). Interest in their application in food and pharmaceutical industries, besides the aquaculture sector, has been increasing (Zárate *et al.*, 2017; Ghasemi Fard *et al.*, 2019; Sprague *et al.*, 2017). Hence, the demand for LC-PUFAs continues to grow (Tocher *et al.*, 2019). Fish oil is the main source of LC-PUFAs and it has been reported that more than 75% of fish oil is used for aquaculture (Tocher, 2015). Hence, the sustainability of the aquaculture system as well as the need to cover the increasing demand necessitate of further available sources for the LC-PUFAs supply. Marine microalgae are considered one of the major natural sources of different compounds including omega-3 PUFA (Adarme-Vega *et al.*, 2012). However, their industrial exploitation is still challenging due to several bottlenecks related to suboptimal cultivation conditions (dissolved CO_2 concentrations and inconsistent light intensities) that result in low cell densities making downstream processing cost-intensive in addition to culture contamination (Chauton *et al.*, 2015). Terrestrial plants such as Camelia and Canola have also been considered and subjected to genetic modification in order to enable the production of n-3 LC-PUFAs. However, genetically engineered plants are subjected to regulatory challenges and very low consumer acceptance in Europe (Ruiz-Lopez 2015; Sprague *et al.*, 2017).

Bacterial production of LC-PUFAs could be a promising alternative to fish oil (Yoshida *et al.*, 2016). Specifically, chemotrophic bacteria can be cultivated in close plants under fully controlled production conditions, which could be optimized reaching promising productivities. Currently, LC-PUFAs production by bacteria has frequently been reported from deep ocean habitats and cold marine environments by relatively low number of bacterial genera. These include for example *Shewanella*, *Colwellia* and *Moritella* (Zhang

and Burgess, 2017; Kautharapu *et al.*, 2013; Wan *et al.*, 2016). To the best of our knowledge, there have been no reports on LC-PUFAs production from Mediterranean Sea.

The aim of this study was to test the ability of 209 isolates obtained from Mediterranean Sea water and sediment samples to produce LC-PUFAs. This is in the perspective of the evaluation of their potential application as feed ingredients in the aquaculture sector.

3.3 Materials and methods

Bacterial isolation

Isolation of the marine bacteria was performed from sediment samples collected from three sites located in the south of Italy by spreading serial dilutions of grinded sediment samples in sterile 3% saline solution on agar plates of modified mineral salt medium (mMSM) containing 1% (w/v) of glucose as the major carbon source (Raddadi *et al.*, 2017). The plates were incubated at 30 °C and the bacterial isolates were purified after three successive streakings on the same medium.

Primary screening of the isolates for LC-PUFAs production using TTC

A primary screening for LC-PUFAs production was performed on bacterial cells after growth in liquid mMSM in 3-ml microtiter plates. After 72 h incubation (30 °C, 150 rpm) 0.1% (w/v) of the dye 2,3,5-triphenyltetrazolium chloride (TTC) was added to the growth broth and the samples were incubated at room temperature for 20 to 30 min. The formation of pink colour was scored as a positive result. Two reference strains, *Moritella marina* DSM 104096 and *Shewanella pacifica* DSM 15445, were used as (+) controls for DHA (Kautharapu *et al.*, 2013) and EPA (Ivanova *et al.*, 2004) production, respectively.

ITS typing and bacterial identification

Dereplication of the bacterial isolates that showed positive results in the TCC colorimetric assay was performed by amplification of the ITS between the 16S and the 23S rRNA genes (ITS-PCR) following the protocol (Cardinale et al., 2004).

For bacterial identification, PCR amplification of 16S rRNA gene was performed on DNA extracted by boiling using the bacterial universal primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1494r (5'-CTACGGCTACCTTGTTACGA-3') with the following reaction conditions: 1× PCR buffer (Invitrogen, Milan, Italy), 1.5 mM $MgCl_2$, 0.12 mM of each dNTP, 0.3 μM of each primer, 0.5 U of Taq polymerase in a final volume of 30 μL. Initial denaturation at 94 °C for 5 min was followed by 5 cycles consisting of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 2 min, and by 30 cycles consisting of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min. A final extension at 72 °C for 10 min was added. The amplification products were sequenced by the company Macrogen (South Korea). Sequences were checked for chimeras using DECIPHER software (Wright *et al.*, 2012),

identified using the BLASTn and a Neighbour-Joining phylogenetic tree was then built using MEGA 6 (Tamura *et al.*, 2013), computing the evolutionary distances using the Kimura 2-parameter model. Sequences were deposited in the GenBank database under accession numbers MN602818 to MN602833.

Lipid extraction and analysis of LC-PUFAs content by GC-FID

The isolates that exhibited a positive result in the TTC reduction assay, different ITS profiles and that were quick to growwere selected for the preparation of fatty acid methyl esters (FAMEs) and gas chromatography (GC) analysis. In addition, 16 isolates among those that were not able to exhibit the pink colour were also selected for the evaluation of their LC-PUFAs production ability by GC analysis.

In a first experiment, bacterial isolates were streaked on mMSM agar and incubated for 15 days at 30 °C. Afterwards, 100 mg of wet biomass were recovered from the surface of the agar plates by a sterile loop and placed in a pre-weighed Pyrex screw cap test tube for FAMEs preparation. In a second set of experiments, a loopful of each bacterial isolates grown on agar plate was used to inoculate a flask containing 20 mL of mMSM. The preculture flasks were incubated on an orbital shaker at 150 rpm and 30 °C for 72 h. Precultures were then employed as inoculum in shake flasks containing 250 mL of mMSM adjusted to pH 8 for 96 h (150 rpm, 30 °C). The bacterial cells were then recovered by centrifugation (14087 rcf, 15 min, 4 °C), washed with a 3% w/v NaCl solution and 100 mg of wet biomass used for FAMEs preparation.

Fatty acid extraction and methylation were performed as described by Masood *et al.* (2005) with some modifications. Lipids were extracted at 100 °C for 1 h in 3.6 mL of 3.3% (v/v) sulphuric acid in methanol. As an internal standard, 200 ppm of benzoic acid was added. After cooling to room temperature, 1.5 mL of n-hexane analytical grade was added to the tubes and vortexed; the upper organic phase containing the FAMEs was collected with Pasteur pipette into a clean vial and used for GC analysis. FAMEs were analyzed by gas chromatography, Agilent Technologies, 9860N) equipped with a Agilent Technologies 7683 Series injector, a flame ionization detector (FID) and an HP-5 column (Agilent J&W GC Columns, part number 19091J-413, 30 m × 320 μm × 0.25 μm), using the method reported by Kumar et al (2014) with some modifications. Nitrogen was used as carrier gas (10 bars). The temperature program for GC was as follows: starting temperature 160 °C; final temperature 270 °C and ramp 10 °C/minute. The run was held at 270 °C for 5 minutes. FAMEs were identified by comparison of retention times with standard mixture (Supelco 37

Component FAME Mix certified reference material, TraceCERT®, in dichloromethane, Sigma-Italy). The relative amount of FAME was quantified by comparing each peak area to the standard.

3.4 Results and Discussion

Screening of the marine isolates

A collection of 209 bacterial isolates obtained from marine sediments collected in three bours in the Mediterranean Sea were tested for potential ability to produce LC-PUFAs using the TTC colorimetric method. In this fast screening assay, the reduction of 2,3,5-triphenyltetrazolium chloride (TTC) from a colourless to a pink/red-triphenyl formazan (TPF) has been reported as an indication of the formation of LC-PUFAs (Ryan *et al.*, 2010; Abd El Razak *et al.*, 2014), since such reduction would be catalysed by enzymes involved in the PUFAs biosynthesis (Ryan *et al.*, 2010). After bacterial growth in liquid mMSM for 72 h, 95 isolates were able to reduce TTC to TPF and were hence considered as potential LC-PUFAs producers. Indeed, further analysis are required in order to confirm the ability of the isolates to produces LC-PUFAs since TTC colorimetric screening has been reported to give false-positive results (Abd El Razak *et al.*, 2014).

Identification of the TTC positive marine isolates

95 isolates positive at coulorimetric assay were dereplicated by ITS typing resulting in 48 different haplotypes (data not shown). Among them, 31 isolates quickly growing were selected for molecular identification by sequencing the 16S rRNA gene and comparing the sequences to the 16S rRNA database. Almost all isolates were from the phylogenetic group Proteobacteria and only very few belonged to Firmicutes group (Table 3.1). Specifically, the gammaproteobacteria were represented by 19 *Marinobacter* sp. (M27.30, P16.20, G1.30, M16.30, G16.20, M3.20, M10.30, M26.30, M13.30, M11.20, M17.30, M24.30, M13.20g, M2.30, M25.30, M14.30, M18.20, M28.20, G19.30) and 4 *Halomonas* sp. (P11.20, M23.30, G7.20, G3.20); the alfaproteobacteria by 4 *Thalssospira* sp. (G9.30, G2.30, P4.20, P17.20) and the Firmicutes by 4 Bacillus sp (M19.20, M20.30, M10.20 and M26.20).

Among these 31 isolates, 15 have been characterized previously. These include G2.30, P11.20 and G3.20 which showed the highest sequence similarities of 99% with *Thalassospira xiamensis, Halomonas titanicae, Halomonas alkaliantarctica* respectively; and other 12 isolates belonging to the genus *Marinobacter* (Raddadi *et al.*, 2017). The remaining 16 isolates, reported for the first time in this study, include: i) M20.30, M26.20, M10.20 and M19.20 which have *Bacillus hwajinpoensis* and *Bacillus hemicentroti* as closest relative type strains; ii) G9.30, P4.20 and P17.20 with sequence similarities of 99% with

Thalassospira permensis (G9.30) and 100% with *Thalassospira xiamensis* (P4.20 and P17.20), iii) M23.30 and G7.20 having a sequence similarity of 99% with *Halomonas titanicae* BH1 and iv) 7 *Marinobacter* sp. isolates. The 16S rRNA gene sequences of isolate G16.20 showed similarity of 99% with *Marinobacter similis* strain A3d10. Isolates M3.20, P16.20 and M13.20 showed a sequence similarity of 99% with *Marinobacter algicola* strain DG893; M2.30 had 100% sequence similarity with *Marinobacter flavimaris* while isolates M14.30 and M11.20 had 99% sequence similarity with *Marinobacter sediminum* and *Marinobacter guineae*, respectively. Despite of this high sequence similarity, it is not possible to identify the isolates to the species level based only on 16S rRNA gene sequences (Ng *et al.*, 2014) and further analyses will be needed in order to establish the exact phylogenetic positions of the isolates.

Table 3.1 List of the 31 marine isolates exhibiting the ability to reduce TCC after growth for 72 h in mMSM at pH 8 with 1% (w/v) glucose as carbon source.

Isolate ID	16SrDNA Accession No	Closest type strain (GenBank Accession No)	16S rDNA identity (%)	Reference
G3.20	MF382058	*Halomonas alkaliantarctica strain CRSS (NR_114902.1)*	99	Raddadi et al (2017)
P11.20	MF382061	*Halomonas titanicae BH1 (NR_117300)*	99	Raddadi et al (2017)
G2.30	MF382062	*Thalassospira xiamenensis DSM 17429 (CP004388)*	99	Raddadi et al (2017)
G1.30	MF382065	*Marinobacter hydrocarbonoclasticus ATCC 49840T (NR_074619)*	99	Raddadi et al (2017)
G19.30	MF382076	*Marinobacter similis strain A3d10 (KJ547704)*	99	Raddadi et al (2017)
M18.20	MF382073	*Marinobacter salarius strain R9SW1(KJ547705)*	99	Raddadi et al (2017)
M28.20	MF382075	*Marinobacter salarius strain R9SW1(KJ547705)*	99	Raddadi et al (2017)
M10.30	MF382067	*Marinobacter guineae strain M3B (NR_042618.1)*	99	Raddadi et al (2017)
M13.30	MF382069	*Marinobacter sediminum strain R65 (NR_029028.1)*	99	Raddadi et al (2017)
M17.30	MF382077	*Marinobacter sediminum strain R65 (NR_029028.1)*	99	Raddadi et al (2017)
M24.30	MF382078	*Marinobacter sediminum strain R65 (NR_029028.1)*	99	Raddadi et al (2017)
M25.30	MF382070	*Marinobacter sediminum strain R65 (NR_029028.1)*	99	Raddadi et al (2017)
M26.30	MF382068	*Marinobacter guineae strain M3B (NR_042618.1)*	99	Raddadi et al (2017)
M27.30	MF382079	*Marinobacter salarius strain R9SW1(KJ547705)*	96	Raddadi et al (2017)
M16.30	MF382055	*Marinobacter salarius strain R9SW1(KJ547705)*	99	Raddadi et al (2017)
P17.20	MN602833	*Thalassospira xiamenensis DSM 17429 (CP004388)*	100	This study
P16.20	MN602821	*Marinobacter algicola strain DG893 (NR_042807.1)*	99	This study
G16.20	MN602832	*Marinobacter similis strain A3d10 (KJ547704)*	99	This study
M23.30	MN602823	*Halomonas titanicae BH1 (NR_117300)*	99	This study
M3.20	MN602829	*Marinobacter algicola strain DG893 (NR_042807.1)*	99	This study
M11.20	MN602825	*Marinobacter guineae strain M3B (NR_042618.1)*	99	This study
M19.20	MN602831	*Bacillus hemicentroti strain JSM 076093 (NR_109010.1)*	98	This study
G7.20	MN602828	*Halomonas titanicae BH1 (NR_117300)*	99	This study
M10.20	MN602826	*Bacillus hwajinpoensis strain SW-72 (NR_025264.1)*	99	This study
M13.20g	MN602824	*Marinobacter algicola strain DG893 (NR_042807.1)*	99	This study
M2.30	MN602827	*Marinobacter flavimaris strain SW-145 (NR_025799.1)*	100	This study
G9.30	MN602830	*Thalassospira permensis strain SMB34 (NR_116841.1)*	99	This study
M26.20	MN602819	*Bacillus hwajinpoensis strain SW-72 (NR_025264.1)*	99	This study
P4.20	MN602822	*Thalassospira xiamenensis DSM 17429 (CP004388)*	100	This study
M20.30	MN602820	*Bacillus hwajinpoensis strain SW-72 (NR_025264.1)*	99	This study
M14.30	MN602818	*Marinobacter sediminum strain R65 (NR_029028.1)*	99	This study

Quantification of the LC-PUFAs

The 31 isolates selected were subjected to lipid extraction, FAMEs preparation and GC-FID analysis in order to confirm their ability to produce LC-PUFAs.

FAMEs were identified by comparing their retention times with those of a FAME standard mixture (FAME mix C4-C24, Sigma-Aldrich) chromatographed in the same conditions and on the same day. The relative amount of individual FAME was quantified by comparing each peak area to the standard. A total of 32 FAME out of the 37 FAME listed by the manufacturer for the 37-component FAME mix were identified using the GC method reported here. All monounsaturated fatty acid esters of the mix (Myristoleic acid, cis-10-Pentadecenoic acid, Palmitoleic acid and cis-10-Heptadecenoic acid) were eluted individually while some polyunsaturated FAMEs were co-eluted possibly due to the limitations of the column/pressure applied here. These correspond to i) α-Linolenic Acid, Linolelaidic Acid, γ-Linolenic Acid, Oleic Acid and Elaidic Acid; ii) Eicosadienoic Acid, Eicosenoic Acid, Eicosatrienoic Acid and iii) Arachidonic Acid (ARA) and Eicosapentaenoic Acid (EPA). It was, however, possible to achieve the separation/individual elution of DHA.

A total of 15 isolates among the 31 that exhibited a positive result in the TCC assay were confirmed to be LC-PUFAs producers, after growth on solid or in liquid mMSM (Figure 3.1). We have found that when cells were grown on mMSM agar plates for two weeks at 30 °C, 12 isolates were confirmed to be LC-PUFAs producers. These correspond to 8 *Marinobacter* sp. isolates (P16.20, G1.30, M16.30, G16.20, M3.20, M10.30, M26.30, M13.30), 2 *Halomonas* sp. isolates (P11.20, G3.20) and 2 *Thalassospira* sp. isolates (G2.30, P17.20). Specifically, GC-FID analysis allowed the detection of DHA production by all isolates. The amount of DHA produced, expressed as mg/g wet cell weight (WCW), varied among the different isolates tested. In particular, three isolates i.e. *Marinobacter* sp. G16.20, *Marinobacter* sp. P16.20 and *Marinobacter* sp. G1.30 produced DHA in significant amounts i.e. 1.43±0.287 mg/g; 0.82±0.164 and 0.63±0.126 mg/g WCW, respectively (Figure 3.1). Under the same conditions, no significant amounts of EPA were detected.

When cells were grown in liquid medium for 96 h at 30 °C, 13 isolates were confirmed to be capable of LC-PUFAs production (Figure 3.1). These include 8 *Marinobacter* sp. isolates (P16.20, G1.30, M16.30, G16.20, M17.30, M24.30, M13.30; M25.30), 2 *Halomonas* sp. isolates (P11.20, G3.20) and one *Thalassospira* sp. isolate P17.20. The highest DHA amount, i.e. 1.85±0.37 mg/g WCW was produced by *Marinobacter* sp. G16.20 (Figure 3.1)

and was higher compared to that detected from the wet biomass of the reference strain *Moritella marina* DSM 104096 (DHA yield of 0.264±0.097 mg/g WCW was achieved).

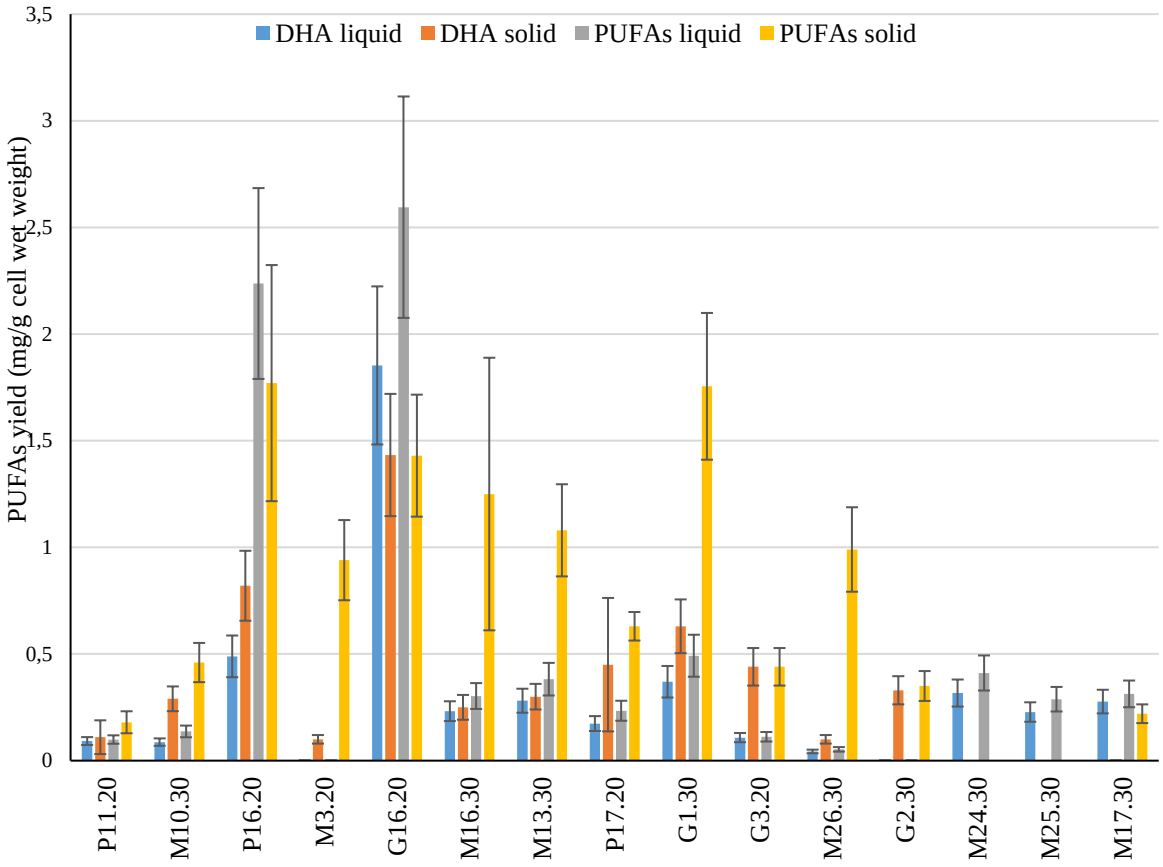

Figure 3.1 Quantitative yields of whole cell PUFAs and DHA in the 15 marine isolates grown in mMSM at 30 °C on solid or liquid medium.

So far, bacterial-derived LC-PUFAs were reported mainly from Gram-negative bacteria belonging to the bacterial phyla Gammaproteobacteria (e.g., *Shewanella*, *Moritella*, *Colwellia*, *Photobacterium*, *Vibrio*) (Shulse and Allen, 2011; Dailey *et al.*, 2015; Moi *et al.*, 2018) and Bacteroidetes (e.g. *Flexibacter*, *Pibocella*, *Cellulophaga*, *Polaribacter* and *Psychroserpens*) (Bianchi *et al.*, 2014). DHA production has been reported from *Moritella marina* (Kautharapu *et al.*, 2013) and *Colwellia psychrerythraea*. Within genus *Colwellia*, the strain *Colwellia psychrerythraea* 34H has been reported to produce mainly DHA but also traces of EPA (Hashimoto *et al.*, 2015).

The amount of LC-PUFAs produced by the isolates in this study is comparable to the natural productivities of other bacteria. For example, the amount of EPA produced by Vibrio sp., in standard artificial sea water medium prior to optimisation was 1.2 mg/g cell dry weight

representing 2% (w/w) of the total fatty acids (Abd El Razak *et al.*, 2014). Similar or even lower productivities were also reported from Antarctic marine bacterial isolates such as *Shewanella* sp. strain 8-5, *Cellulophaga*, *Pibocella* and *Polaribacter* (Bianchi *et al.*, 2014). On the opposite of what have been reported in a previous study (Abd El Razak *et al.*, 2014) where among 81 screened bacterial isolates none was able to produce LC-PUFAs, Mediterranean Sea bacterial isolates are able to produce DHA. Although comparison of the results could not be performed since the authors did not report the identity of the isolates, the different results could be due to different isolation/cultivation conditions as well as to different microniches as has been suggested by Shulse and Allen (2011).

In this study, among 31 isolates resulting in a positive TCC response and quickly growing, a maximum of 15 isolates (depending on the growth conditions in solid or liquid media) were confirmed to be LC-PUFAs producers. This indicates that almost 50% of TCC results were false-positive, confirming data reported previously about using the colorimetric assay for the screening (Abd El Razak *et al.*, 2014). On the other hand, among the 40 isolates that resulted negative to the TCC assay, 16 were screened using GC-FID to confirm the validity of TTC negative screening result. None of these TCC-negative isolates was shown to produce LC-PUFAs, meaning that the TCC assay resulted in 0% false-negative as has been reported previously (Abd El Razak *et al.*, 2014). Also, TCC has been reported as a useful assay for the screening of EPA production, its usefulness for a rapid screening of DHA production has been confirmed in this study.

Time course of cell growth in mMSM broth and FA production by isolate *Marinobacter* sp G16.20

Marinobacter sp. isolate G16.20, which was shown to produce the highest DHA concentration among all the isolates screened here, was selected for further evaluation of its growth and productivity for a growth time of up to one week (216 h). The results obtained are shown in figure 3.2. The increase in total fatty acids and DHA content matches that of cell growth. After 24 h cultivation, exponential bacterial growth was started and the cells entered the stationary phase after 96 h. GC-FID analyses allowed the detection of low concentrations of DHA after 24 h incubation which increased with increasing incubation time. At the beginning of the stationary phase, i.e. after 96h cultivation, the highest DHA titre achieved corresponds to 1.85±0.371 mg/g WCW was (Figure 3.2). Further prolongation of the incubation time to 168 h did not result in significant improvement of productivity and

a DHA yield of 1.91±0.382 mg/g was recorded. Such DHA yield amounts for 45.89% of the total fatty acids identified using the method reported here, in mMSM at 30 °C for after 4 days. Similar productivities have been reported for GC-FID analysis of EPA produced by *Shewanella putrefaciens* which represented 24-40% of the total fatty acid in the cell (Yazawa, 1996). These values are higher compared to those reported from other DHA-producing bacteria (Bianchi *et al.*, 2014). Finally, when the incubation period was extended to 216 h, a decrease of the productivity was observed, which is most probably due to the bacterial cell lysis/death as a result of the depletion of nutrient sources.

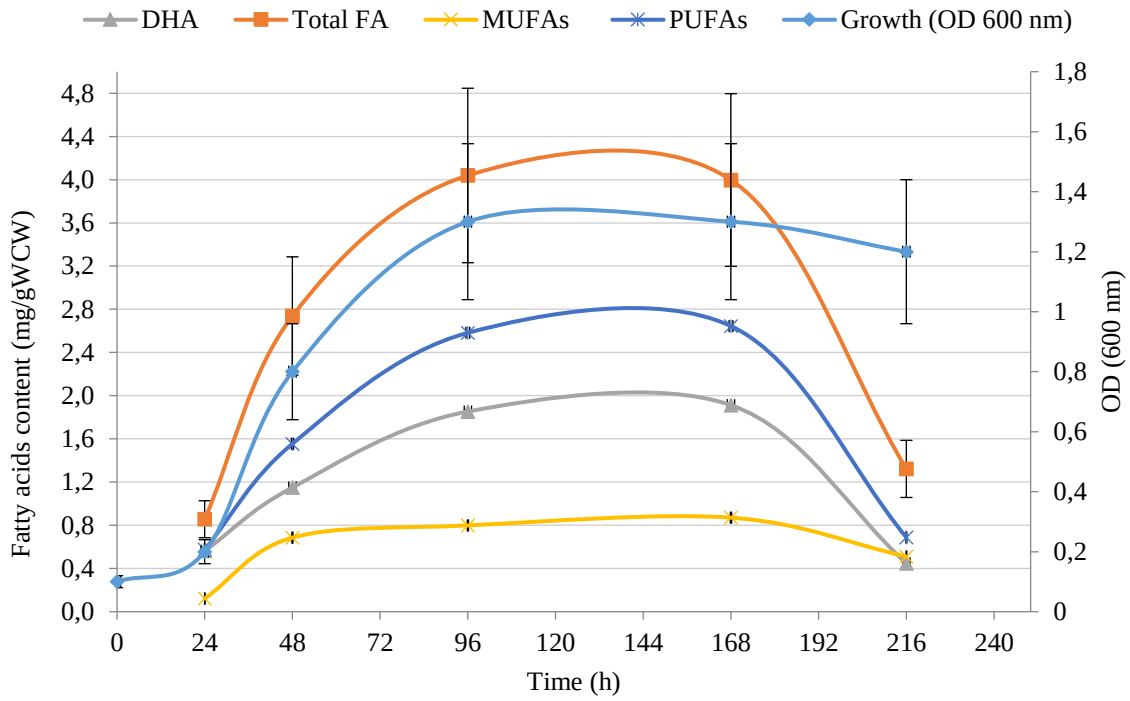

Figure 3.2 Time course of cell growth and quantitative yield of whole cell TFAs, MUFAs, PUFAs and DHA in isolate *Marinobacter sp.* G16.20 grown in mMSM at 30 °C during the time period. Total fatty acids (TFAs); docosahexaenoic acid (DHA); monounsaturated fatty acids (MUFAs); polyunsaturated fatty acids (PUFAs); growth (OD_{600nm}). The experiments were carried out in triplicate and values are means of three samples.

3.5 Conclusion

Among 209 marine bacterial isolates from the Med Sea, 31 were selected as potential LC-PUFAs producers based on their ability to reduce TCC to TPF, ITS typing and quickly growing were subjected to further analyses. The isolates, identified by 16S rRNA gene analysis, were Gram-negative and Gram-positive bacteria representatives of *Proteobacteria* and *Firmicutes*. GC-FID analysis of FAMEs prepared from these isolates showed that only *Proteobacteria* were able to produce LC-PUFAs, particularly DHA, after growth at 30 °C. This is of interest in the perspective of a large-scale production and purification of DHA; since extra costs due to cooling (in the case of the use of psychrophilic DHA-producing bacteria for example) would be avoided.

Different *Marinobacter* sp., *Thalassospira* sp and *Halomonas* sp. isolates could produce DHA. Production of LC-PUFAs from these genera has not been reported previously, to the best of our knowledge. All isolates from this study produced relatively low levels of DHA compared with levels produced by strains in other genera such as *Colwellia*. Nevertheless, considering that bacteria from the genus *Marinobacter* have never been reported to be involved in human or animal pathology, strain *Marinobacter* sp G16.20 has the potential to be used as an LC-PUFAs source ingredient for the preparation of feed in aquaculture sector, after production process optimization.

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4. Antimicrobial compounds active against aquaculture fish pathogenic bacteria produced by *Marinobacter* spp.

4.1 Abstract

The massive use of antibiotics in the medical and veterinary fields has led to the emergence of antibiotic resistant bacteria also in aquaculture environments and the increase of antimicrobial resistance in fish pathogens. Hence, the discovery of friendly alternatives to the use of antibiotics is of great interest for the sustainability of the aquaculture sector. In this context, bacteriocin-like substances appear to be an excellent candidate and marine bacteria could be a valuable source for the production of new highly active antimicrobial substances.

The aim of this study was, hence, to select marine bacteria able to produce antimicrobial compounds active against four bacterial species associated to different diseases of farmed fish and to evaluate the stability of the produced compounds. Among 41 marine isolates screened, none inhibited the growth of *F. psychrophilum* while 35, 17 and 4 exhibited antibacterial activity towards *Y. ruckeri*, *P. damselae* and *V. harveyi*, respectively. Interestingly, 4 isolates inhibited all three pathogens and 17 were able to inhibit two indicator strains. Among these, 8 marine isolates produced antimicrobial compounds that exhibited 100% residual activity after exposure to pH/temperature extremes and after proteinase K treatment. These results provide interesting inputs for further studies dedicated to the recovery of these active compounds, the elucidation of their nature and evaluation of their effectiveness in inhibiting fish pathogens in rearing conditions in order to use them as friendly alternatives to antibiotics for more sustainable aquaculture sector.

***Keywords*:** antimicrobial compounds, fish pathogen, sustainable aquaculture, *Marinobacter* sp.

4.2 Introduction

Aquaculture is a food-producing sector that accounts for nearly 50% of the food fish market in the world (EUMOFA, 2016; FAO, 2018). However, the development of this sector has been associated with environmental, social and economic issues due to management costs, massive use of drugs against fish diseases, fish feed disponibility and production of waste and by-products. For the sustainability of aquaculture sector measures for the reduction of these problems, with particular focus on solutions for the reduction of drugs use are needed (Subasinghe *et al.*, 2009).

In fact, 10% of all cultured fish are lost due to diseases of bacterial origin (Adams, 2019). After the discovery of penicillin in 1928, antibiotics have become a crucial tool in the medical field and are used as feed additives in cattle, poultry, pigs (Van Boeckel *et al.*, 2015; Bacanli *et al.*, 2019) and in aquaculture for improving the growth of the animals and for the treatment of diseases of bacterial origin (Henriksson *et al.*, 2015). However, the massive use of antibiotics in aquaculture has negative effects on the aquatic environment and increases the risks associated with the development of antimicrobial resistance by fish pathogens. (Saga *et al.*, 2009; Davies *et al*, 2010; Romero *et al.*, 2012).

Consequently, it is necessary to develop alternatives solutions to the use of classic antibiotics (Suresh *et al.*, 2018) and search new antimicrobial compounds with better potential and bioavailability, mild or moderate adverse reactions and minimal toxicity (Pérez *et al.*, 2016).

Vaccines are important tools to reduce use of antibiotics in aquaculture sector. These preparations consist in killed pathogens (or their components) and adjuvants and are usually administered by intraperitoneal injection. Currently, fish vaccines are available for 17 species of fish, including salmonids and marine species (i.e. cod, asian sea bass, flatfish, red sea bream, yellowtail) (Adams, 2019; Brudeseth *et al.*, 2013).

However alternative to the use of antibiotics has limitations: the development of vaccines is not cost effective and hence could not be a solution for most of the aquaculture breeders; and most importantly, this could not be a solution for all fish species since some do not develop a reactive immune system (Henriksson *et al.*, 2018).

In recent years an increasing number of scientists have addressed the search for new natural antimicrobial molecules in the marine environment and in particular those originating from the metabolism of marine microorganisms (Bindiya *et al.*, 2016; Liu *et al.*, 2016; Wang *et al.*, 2018). Marine microorganisms are able to survive in extreme and competitive habitat by producing non-ribosomal secondary metabolites, bacteriocins, lysozymes, proteases,

surfactants/biosurfactants and exopolysaccharides (Baharum *et al.*, 2010; Prieto *et al.*, 2012; Schinke *et al.*, 2017). Bacteriocins have a remarkable specificity of action that distinguishes them from common antibiotics (Gratia, 1946). In addition, bacteriocinogenic bacteria may also engage as probiotics and confer health benefits to the organisms that host them, including farmed aquaculture animals (Gillor, 2008; Nguyen *et al.*, 2014; Hoseinifar *et al.*, 2018; Lopetuso *et al.*, 2019). Antibacterial and antiviral activities of biosurfactants make them important molecules for applications in the fight against many diseases and as therapeutic agents (Harshada, 2014). Exopolysaccharides are carbohydrate polymers that, in some cases, can form a highly hydrated layer around the cells and can be linked to different structural components of the cell. Many researchers have focused on their high molecular weight as a source of potentially bioactive derivatives with antibacterial activity (McEwen *et al.*, 2018).

In the present study, a collection of marine bacteria was screened for the selection of isolates which have the ability to produce antimicrobial compounds, potentially bacteriocins, active against four fish pathogens responsible for diseases in aquaculture. Identifying marine isolates that produce molecules with antibacterial activity, allows controlling the pathogens of fish and representing a valid alternative to the massive use of conventional antibiotics and antimicrobial agents that have negative consequences on environment and human health (Sahoo *et al.*, 2016; Rather *et al.*, 2017; Scott *et al.*, 2018).

4.3 Materials and methods

Bacterial strains and culture conditions

A collection of 41 bacterial isolates obtained from marine environmental as previously reported (Raddadi *et el.*, 2017) were identified (see Chapter 3.3) and used for the screening of their antibacterial activities. The strains were maintained ad -80 °C in modified mineral salt medium (mMSM) supplemented with 20% (v/v) glycerol. Working cultures were grown in mMSM (pH 8) at 30 °C.

Indicator strains and culture conditions

The indicator strains used in this study include four farmed fish pathogenic bacterial species. *Yersinia ruckeri* (DMSZ 18506) (Gibson, 1998), *Vibrio harveyi* (DMSZ 19623) (Desriac *et al.*, 2010), *Photobacterium damselae subsp. Piscicida* (DMSZ 19623) (Touraki *et al.*, 2012) and *Flavobacterium psychrophilum* (DSMZ 3660) (Boutin *et al.*, 2012). *Y. ruckeri* was grownin CY medium, *P. damselae subsp. Piscicida* in Bacto-marine medium (BM) (DIFCO), *V. harvyi* and *F. psychrophilum* in Trypticase Soy Broth (TSB) (Sigma-Aldrich, Steinheim, Germany) as recommended by DSMZ. The strains were maintained frozen (-80 °C) in their corresponding growth media supplemented with 20% v/v glycerol.

Preliminary screening of marine isolates for antimicrobial compounds production

Marine isolates were inoculated into mMSM broth and the flasks were incubated at 30°C for 72 h on rotary shaker (150 rpm). The experiments were performed in 100 mL flask containing 20 mL of broth. After bacterial growth, the cell free supernatant (CFS) were recovered by centrifugation (10000 rpm, 10 min at 4°C) and filtration (0.22 µm cellulose acetate filter membrane). The well-diffusion method was used to examine anti-bacterial activity of CFS against indicator strains (Touraki *et al.*, 2012).

Well-diffusion assay for the determination of antimicrobial activity

Well-diffusion method was used to evaluate the antimicrobial activity of CFS. Analogous to the official disk-diffusion method accepted by Clinical and Laboratory Standards Institute (CLSI), the agar plate surface was inoculated by pouring a volume of soft TSA agar (7.5

g/L) containing 10^5-10^6 CFU/mL of indicator strain (Linton, 1958). Then, a well with a diameter of 10 mm was created with a sterile tube, and 200 µL of CFS has been inserted into it. After 24 h incubation at 30 °C, the presence of clear zone around the well was considered as an evidence of antimicrobial compounds in CFS. The inhibitory zone was measured (cm) (Balouiri *et al.*, 2016). Sterile mMSM pH 8 incubated for 72 h at 30°C and subsequently filtered-sterilized (0.22 µm) was used as negative control.

Kinetic of antimicrobial compounds production

Marine isolates exhibiting, during preliminary screening the ability to produce antimicrobial compounds active against al least two indicator strains were selected for further studies. A loopful of each strain grown on mMSM agar plate was used to inoculate a 100 mL flask containing 20 mL of mMSM broth. The preculture flasks were incubated on an orbital shaker at 150 rpm and 30 °C for 72 h. Active cells from precultures were then employed as inoculum [10% (v/v)] in 1 L conical flasks containing 200 mL of sterile mMSM. Flasks were incubated at 30 °C on an orbital shaker (agitation 150 rpm) for up to 72 h. Samples were aseptically withdrawn after 0, 24, 48 and 72 h incubation for growth monitoring ($O.D._{600\ nm}$) and evaluation of CFS antimicrobial activity by well diffusion assay.

Preliminary characterization of antimicrobial compounds: sensitivity to pH, temperature and proteinase K

CFSs harvested after 24 h incubation were used for antimicrobial compounds characterization. Sensitivity of the antimicrobial compounds to the proteolytic enzyme (Proteinase K, 1 mg/mL), pH (4 and 10) and temperatures (60 °C, 80 °C and 121 °C) were tested and the results were expressed as residual activity (RA %):

$$\text{RA (\%)} = \frac{\text{diameter (cm) of inhibition halo by CFS treated} \cdot 100}{\text{diameter (cm) of inhibition halo by CFS untreated}}$$

The pH sensitivity of antimicrobial compounds was determined by adjusting the CFS to pH 4 and 10 with either 1 N HCl or 1 N NaOH. After 2 h incubation at 30 °C, the pH of CFSs was readjusted to 7 before performing the well diffusion assay. To check the thermal stability of antimicrobial compounds, CFSs were exposed for 15 min to different temperatures (60 °C or 80 °C in heating block, or 121 °C in autoclave). To check the resistance of

antimicrobial compounds to proteolysis, proteinase K (Sigma-Aldrich, Steinheim, Germany) at final concentration of 1 mg/mL (in 100 mM Tris/HCl buffer, pH 7.5) was added to CFSs. Then, the samples were incubated for 1 h at 37 °C (optimum digestion temperature recommended). Following incubation, the enzyme was heat inactivated for 3 min at 100°C. For each assay, untreated CFS plus 100 mM Tris/Cl pH 7.5, CFS plus buffer treated 3 min at 100 °C, 100 mM Tris/HCl pH 7.5 alone and proteinase K 1 mg/mL solutions were used as controls. After all treatments the residual activity was determined by well diffusion assay.

Partial purification of antimicrobial compounds and molecular weight estimation

Partial purification of antimicrobial compounds was detected as described by Cherif *et al.* (2008). Marine isolates were grown pre-grown in mMSM medium pH 8 for 72 h at 30 °C (150 rpm). After the incubation, 10% (v/v) of an inoculum was inoculated into 1 L of sterile mMSM medium pH 8 and incubated at 30°C for 24 h (150 rpm). Then, the cultures were centrifuged at 10000 rpm for 30 minutes (4 °C) and the CFS were precipitated with ammonium sulphate (80 % saturation) for 24 h at 4 °C. Subsequently, the protein precipitated were recovered by centrifugation at 10000 rpm for 30 minutes (4 °C) and dissolved in 100 mM Tris/HCl buffer, pH 7.5. The concentrate solutions were dialysed for 24 h against 4 L of same buffer in Spectra-Por no. 3 dialysis tubing. Partially purified samples were tested by well diffusion assay against *Y. ruckeri*, *V. harveyi* and *P. damselae subsp. Piscicida*.

In order to estimate the approximate molecular mass and evaluate the proteinaceous nature of antimicrobial compounds, partially purified samples were subjected to SDS-PAGE as reported by Ouzari (2008) with some modifications. Polyacrylamide concentration in the stacking and separating gel were 5% and 15% respectively. Duplicates of each samples (20 µL) were loaded simultaneously with the standard proteins (low molecular weight markers). Electrophoresis was conducted at a constant voltage of 50 V for 1 h and at 100 V for 5 h. After electrophoresis, the gel was cut vertically. The first part was stained with Comassie brilliant blue G-250 (Sigma-Aldrich, Steinheim, Germany); as described by Cherif (2008), the second part was assayed by gel overlay assay for direct detection of activity.

4.4 Results and discussion

Preliminary screening

Forty one marine bacterial isolates (Table 4.1), grown in mMSM agar plate and subsequently inoculated into 100 mL flask containing 20 mL of mMSM for 72 h at 30 °C (150 rpm), were screened for the antimicrobial compounds production by well diffusion assay. Among them 35 isolates were able to inhibit *Y. ruckeri* and hence were identified as producers (Figure 4.1). Out of these 35 isolates, 17 were able to inhibit the growth of *P. damselae* and 4 were active towards both *P. damselae* and *V. harveyi*. No inhibitory activity towards *F. psychrophilum* was recorded in CFS all the isolates tested here.

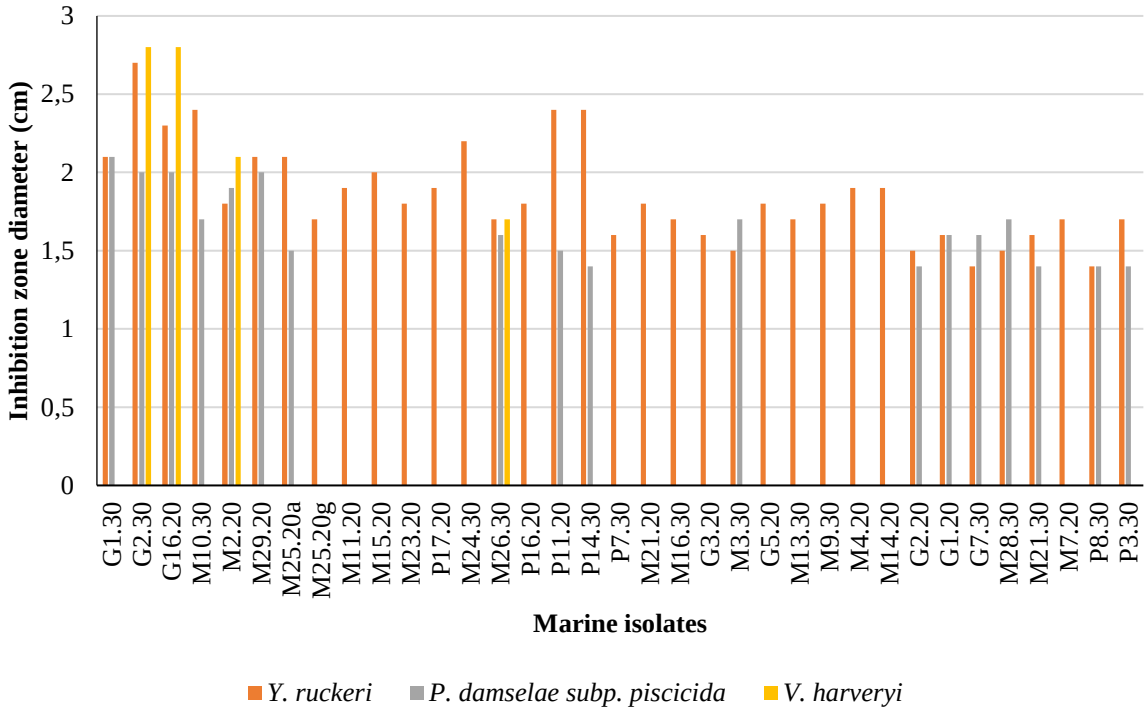


Figure 4.1 Results of preliminary screening of the antimicrobials production by the 41 isolates.

Table 4.1 List of the 41 marine isolates screened for antimicrobial compounds production.

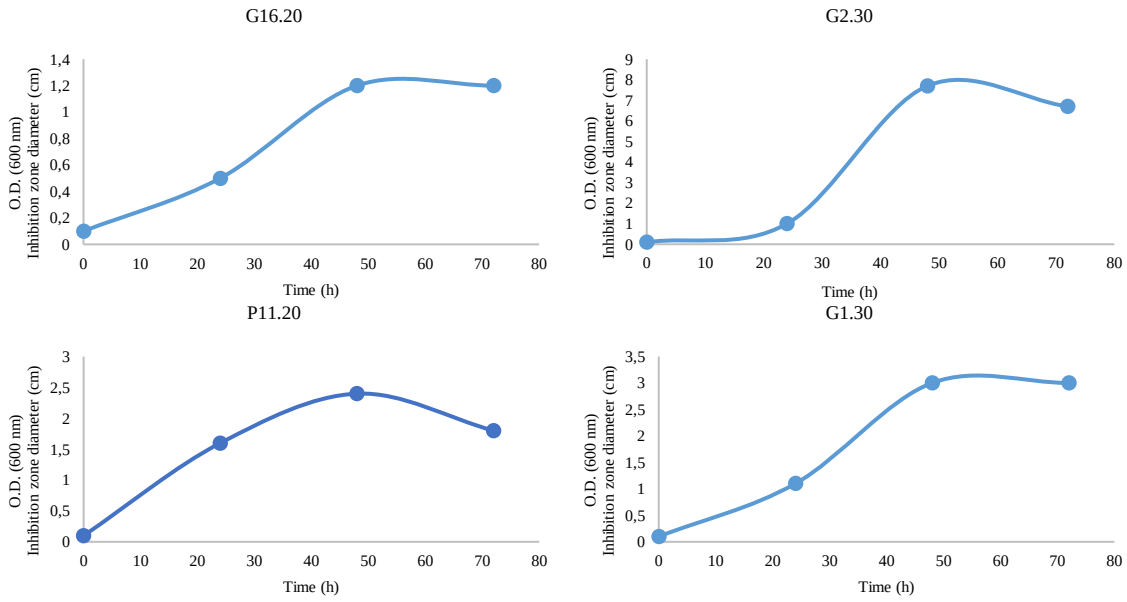
Isolate ID	16S rDNA Accession No	Closest type strain (GenBank Accession No)	16S rDNA identity (%)	Reference
G1.30	MF382065	*Marinobacter hydrocarbonoclasticus ATCC 49840T (NR_074619)*	99	Raddadi et al (2017)
G2.30	MF382062	*Thalassospira xiamenensis DSM 17429 (CP004388)*	99	Raddadi et al (2017)
G16.20	MN602832	*Marinobacter similis strain A3d10 (KJ547704)*	99	This study
M10.30	MF382067	*Marinobacter guineae strain M3B (NR_042618.1)*	99	Raddadi et al (2017)
M2.20	in progress	*Marinobacter salarius strain R9SW1(KJ547705)*	99	
M29.20	in progress	Marinobacter salarius strain R9SW1(KJ547705)	99	
M25.20a	in progress	*Marinobacter salarius strain R9SW1(KJ547705)*	99	
M25.20g	in progress	*Marinobacter salarius strain R9SW1(KJ547705)*	99	
M11.20	MN602825	*Marinobacter guineae strain M3B (NR_042618.1)*	99	This study
M15.20	in progress	Marinobacter salarius strain *R9SW1(KJ547705)*	99	
M23.20	in progress	*Marinobacter guineae strain M3B (NR_042618.1)*	99	
P17.20	MN602833	*Thalassospira xiamenensis DSM 17429 (CP004388)*	100	This study
M24.30	MF382078	*Marinobacter sediminum strain R65 (NR_029028.1)*	99	Raddadi et al (2017)
M26.30	MF382068	*Marinobacter guineae strain M3B (NR_042618.1)*	99	Raddadi et al (2017)
P16.20	MN602821	*Marinobacter algicola strain DG893 (NR_042807.1)*	99	This study
P11.20	MF382061	*Halomonas titanicae BH1 (NR_117300)*	99	Raddadi et al (2017)
P14.30	CP039374.2	*Halomonas titanicae strain ANRCS81*	100	
P7.30	MF382054	*Marinobacter adhaerens strain HP15 (NR_074765)*	100	Raddadi et al (2017)
M21.20	MK493604.1	*Marinobacter algicola strain S12B-101 16S ribosomal RNA, partial sequence*	99	
M16.30	MF382055	*Marinobacter salarius strain R9SW1(KJ547705)*	99	Raddadi et al (2017)
G3.20	MF382058	*Halomonas alkaliantarctica strain CRSS (NR_114902.1)*	99	Raddadi et al (2017)
M3.30		*Marinobacter adhaerens HP15 16S ribosomal RNA, partial sequence*	100	
G5.20	MF382059	Halomonas venusta strain DSM *4743 (NR_042069.1)*	98	Raddadi et al (2017)
M13.30	MF382069	*Marinobacter sediminum strain R65 (NR_029028.1)*	99	Raddadi et al (2017)
M9.30	in progress	*Marinobacter guineae strain M3B (NR_042618.1)*	99	

M4.20	in progress	*Marinobacter salarius strain R9SW1(KJ547705)*		
M14.20	MF401328.1	*Marinobacter sp. strain InAD-124 16S ribosomal RNA gene, partial sequence*	100	
G2.20	MK493588.1	*Marinobacter flavimaris strain H05Y-240 16S ribosomal RNA gene, partial sequence*	99.71	
G1.20	KM279024.1	*Rhodobacteraceae bacterium DG1570 16S ribosomal RNA gene, partial sequence*	97.14	
G7.30	NR_115011.1	*Thalassospira lucentensis MCCC 1A00383 = DSM 14000 strain QMT2 16S ribosomal RNA, partial sequence*	99.24	
M28.30	KJ573108.1	*Marinobacter sp. DS1930-III 16S ribosomal RNA gene, partial sequence*	98	
M21.30	KT758423.1	*Bacillus hwajinpoensis strain HQB224 16S ribosomal RNA gene, partial sequence*	99	
M7.20	in progress	*Marinobacter salarius strain R9SW1(KJ547705)*	99	
P8.30	KT922024	*Thalassospira permensis strain I-A-E-11 16S ribosomal RNA gene, partial sequence*	99	
P3.30	KT922024	*Thalassospira permensis strain I-A-E-11 16S ribosomal RNA gene, partial sequence*	99	
M12.30g	AB305301	*Marinobacter sp. KJ6-1-1 gene for 16S rRNA, partial sequence*	99	
M16.20g	in progress	*Marinobacter salarius strain R9SW1(KJ547705)*	99	
M2.30	MN602827	*Marinobacter flavimaris strain SW-145 (NR_025799.1)*	100	This study
M17.20	MF382057	*Bacillus hwajinpoensis SW-72 (NR_025264)*	99	
M28.20	MF382075	*Marinobacter salarius strain R9SW1(KJ547705)*	99	Raddadi et al (2017)
M13.20g	MN602824	*Marinobacter algicola strain DG893 (NR_042807.1)*	99	This study

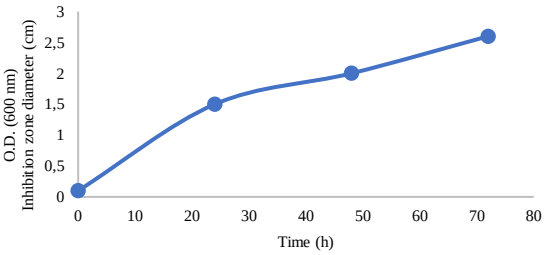
It can be concluded from the diameter of inhibition zone (in cm) observed in Figure 3.1 that 4 marine isolates (M26.30, G2.30, G16.20 and M2.20) produced one or more molecules with antimicrobial activity against *Y. ruckeri*, *P. damselae* and *V. harveyi*. 14 marine isolates (P8.30, G1.30, G1.20, M10.30, M29.20, M25.20a, G2.20, M21.30, P3.30, P11.20, P14.30, M3.30, G7.30 and M28.30) produced one or more molecules with antimicrobial activity against both *Y. ruckeri* and *P. damselae* while 17 isolates (M25.20g, M11.20, M15.20, M23.20, P17.20, M24.30, P16.20, P7.30, M21.20, M16.30, G3.20, G5.20, M13.30, M9.30, M4.20, M14.20 and M7.20) produced one or more molecules with antimicrobial activity only against *Y. ruckeri*.

Growth and kinetic of antimicrobial compounds production

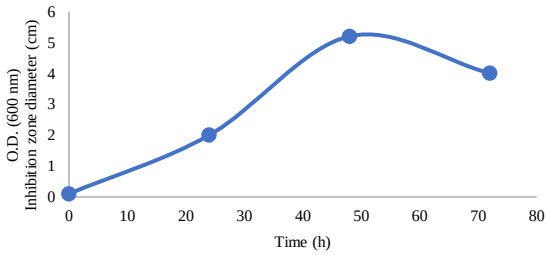
Amount the 35 marine isolates identified as antimicrobial compounds producers in the preliminary screening, 17 exhibiting inhibitory activity towards at least 2 indicator strains were selected for further studies. Pre-cultures of these isolates were obtained from single bacterial colonies transferred from mMSM agar plates into 100 mL flasks containing 20 mL of mMSM and grown for 72 h at 30°C (150 rpm). Subsequently, 10% (v/v) of pre-culture was added to 1 L flasks containing 200 mL of the same medium and incubated at 30 °C (150 rpm). At time 0 (T0) and intervals of 24 h samples were aseptically withdrawn and used for bacterial growth ($O.D._{600\ nm}$) and antimicrobial activity of CFS evaluation. As reported in Figure 4.2, almost all marine isolates reached exponential growth phase between 0 and 48 h incubation. Exception is mode for isolates M26.30, M29.20, M25.20a, P16.20 and M25.20g, which reached its exponential growth phase after 72 h incubation.



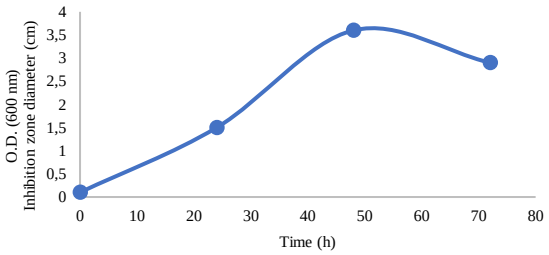
P16.20



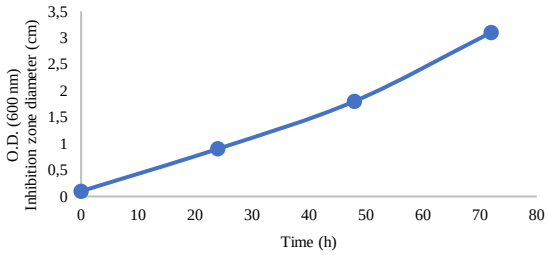
P17.20



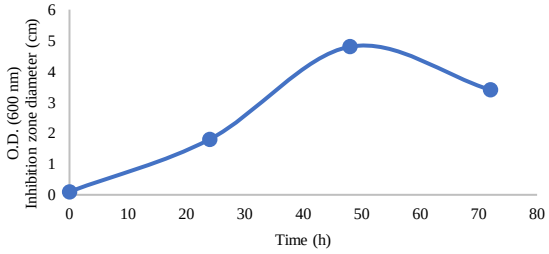
M24.30



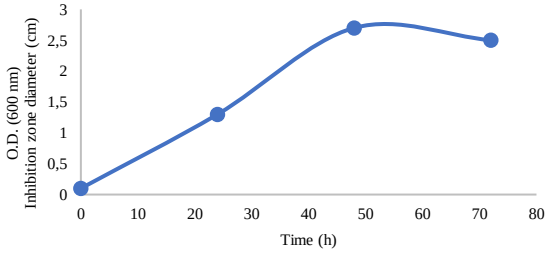
M25.20g



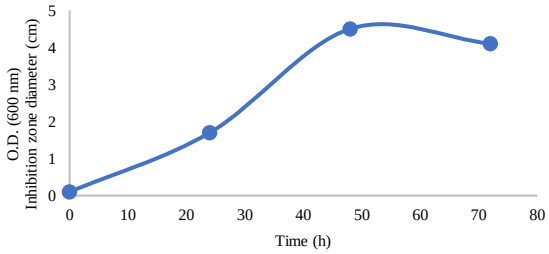
M10.30



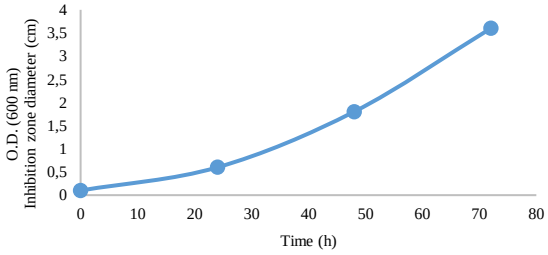
M15.20



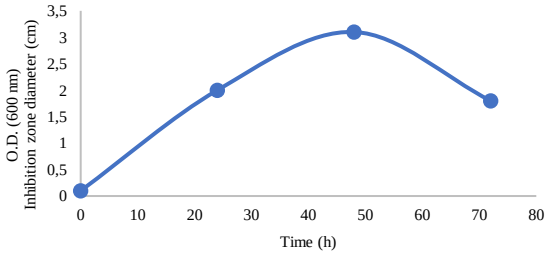
M23.20



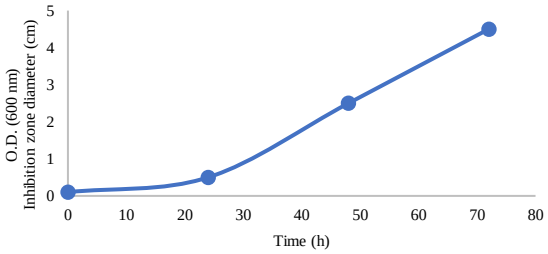
M26.30



P14.30



M25.20a



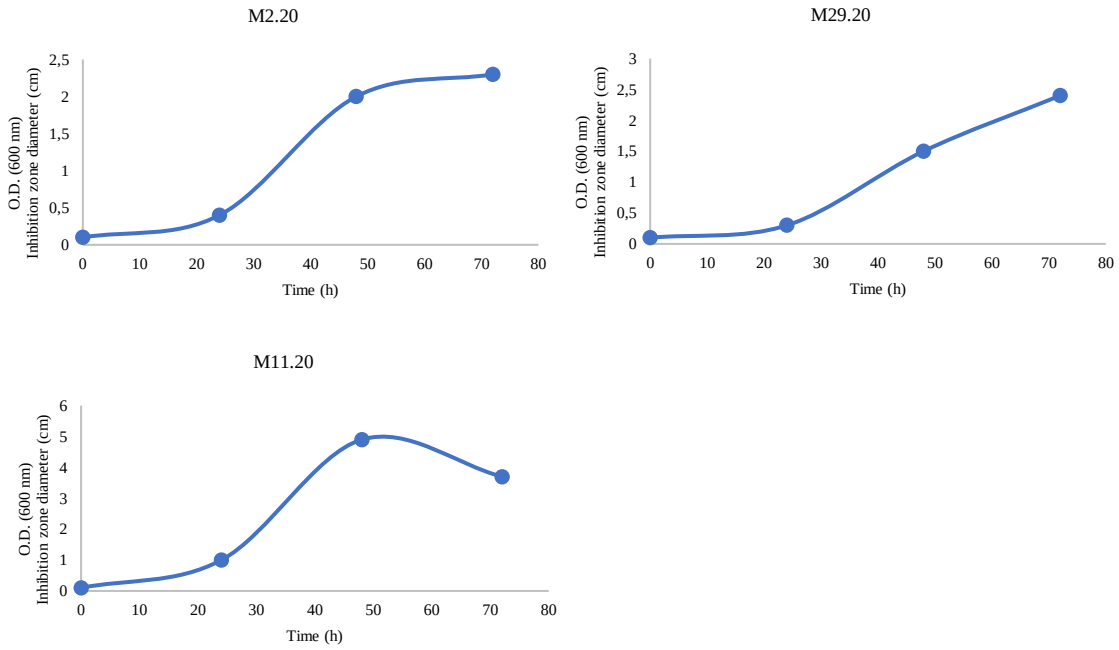


Figure 4.2 Growth kinetics of the 17 marine isolates (O.D. 600 nm) able to inhibit at least 2 indicator pathogens.

As reported in Table 4.2 16 CFS of 17 marine isolates (except for M11.20 isolate) displayed antimicrobial activity against at least two indicator strains (*Y. ruckeri* and *P. damselae*) at T0. At same time, only the CFS of 7 marine isolates (P16.20, P17.20, M24.30, M11.20, M25.20g, M15.20 and M23.20) showed antimicrobial activity against *V. harveyi*. These data suggest the production of antimicrobial compounds in pre-cultures and highlight the high activity/amount of the produced compounds allowing the detection of the inhibitory effect towards the indicator strains even after dilution (through the transfer from pre-culture to culture).

Table 4.2 Results of the 17 isolates selected based on the results of the preliminary screening and tested for the production of antibacterial molecules. -: no inhibition zone; +: diameter of inhibition zone 0-1.5 cm; ++: diameter of inhibition zone 1.5-2 cm; +++ diameter of inhibition zone 2-4.3 cm.

Indicator strains	***Y. ruckeri***				***P. damselae***				***V. harveyi***			
Marine isolates	**T0**	**24 h**	**48 h**	**72 h**	**T0**	**24 h**	**48 h**	**72 h**	**T0**	**24 h**	**48 h**	**72 h**
G1.30	+++	+++	++	-	++	+++	-	-	-	++	-	-
G2.30	+++	++	++	-	+++	+++	-	-	-	++	-	-
G16.20	+++	+++	+	-	++++	+++	-	-	-	+++	-	-
P11.20	+++	++	-	-	++	+++	-	-	-	-	-	-
M10.30	+++	++	-	-	+++	+++	-	-	-	++	-	-
P14.30	++	+	-	-	++	++	-	-	-	-	-	-
M2.20	++	++	+	-	++	+++	++	-	-	++	-	-
M26.30	++	++	+	-	+++	+++	+	-	-	++	-	-
M29.20	++	++	+	-	++	+++	+	-	-	+++	-	-
M25.20a	++	++	+	-	+++	+++	++	+	-	++	-	-
P16.20	++	++	++	++	+	+	-	-	+	-	-	-
P17.20	+	++	++	++	++	-	-	-	+++	-	-	-
M24.30	+	++	++	+	+++	++	-	-	++	++	-	-
M25.20g	++	+	+	++	++	++	-	-	++	+	-	-
M11.20	-	+	+	+	++	-	-	-	+	-	-	-
M15.20	++	++	++	+	+++	+	-	-	+	-	-	-
M23.20	+	+	+	+	++	++	-	-	++	-	-	-

Evaluation of the antimicrobial activity of the CFS recovered at the following sampling points i.e. after 24 h, 48 h and 72 h incubation, revealed behaviour towards the indicator strains.

Well diffusion assay showed (Figure 3.3 and Table 4.2) that *Y. ruckeri* was inhibited by CFS of 17 marine isolates after 24 h incubation. Only 7 of them showed a relatively constant inhibitory activity up to 72 h. For the remaining isolates (G1.30, G2.30, G16.20, M26.30, P11.20, M10.30, P14.30, M2.20, M29.20, M25.20a), a decrease of the antimicrobial effect up to complete absence after 48 h or 72 h was observed.

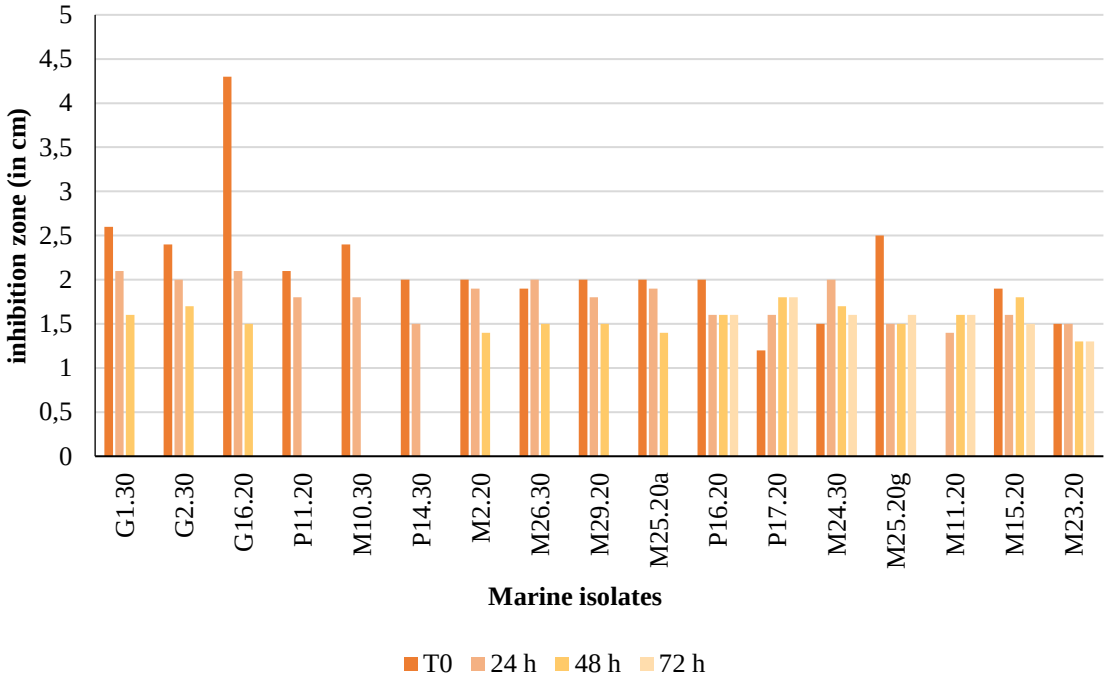


Figure 4.3 Kinetic of production of antimicrobial compounds active against *Y. ruckeri* expressed as variation of inhibition zone (in cm).

These results suggest that for these later isolates, the production of antimicrobial compounds active against this indicator strains occurred only in pre-culture and that the decrease of the antimicrobial effect by increasing the incubation time could be due to degradative processes during the culture growth and/or inactivation of the antimicrobial compounds as a result of pH change, at variation of gene expression or at inactivating complex formation with other extracellular products (Paik *et al.*, 1997).

CFS of P16.20, M15.20 and M23.20 marine isolates showed constant antimicrobial activity at T0, 24 h, 48 h and 72 h, suggest the stability of antimicrobial compounds; CFS of M25.20g marine isolate displayed high antimicrobial activity at T0 which decreases in the time; CFS of P17.20, M24.30 and M11.20 marine isolates exhibited an increase of antimicrobial activity in the time suggest the production of one or more antimicrobial compounds during the growth of marine isolates.

Data obtained against *P. damselae* (Figure 4.4 and Table 4.2) highlighted that it was inhibited by CFS of 15 marine isolates recovered after 24 h incubation. Antimicrobial activity was observed in the CFSs recovered after 48 h of 4 isolates and only one isolate at the end of the experiment. Hence also for this indicator strain the general trend was a decrease of the antibacterial effect with the increase of the incubation time suggesting the production of antimicrobial compounds active against *P. damselae* only in precultures. Such decrease could be due to biodegradative process or inactivation of the antimicrobial compounds during the microbial growth.

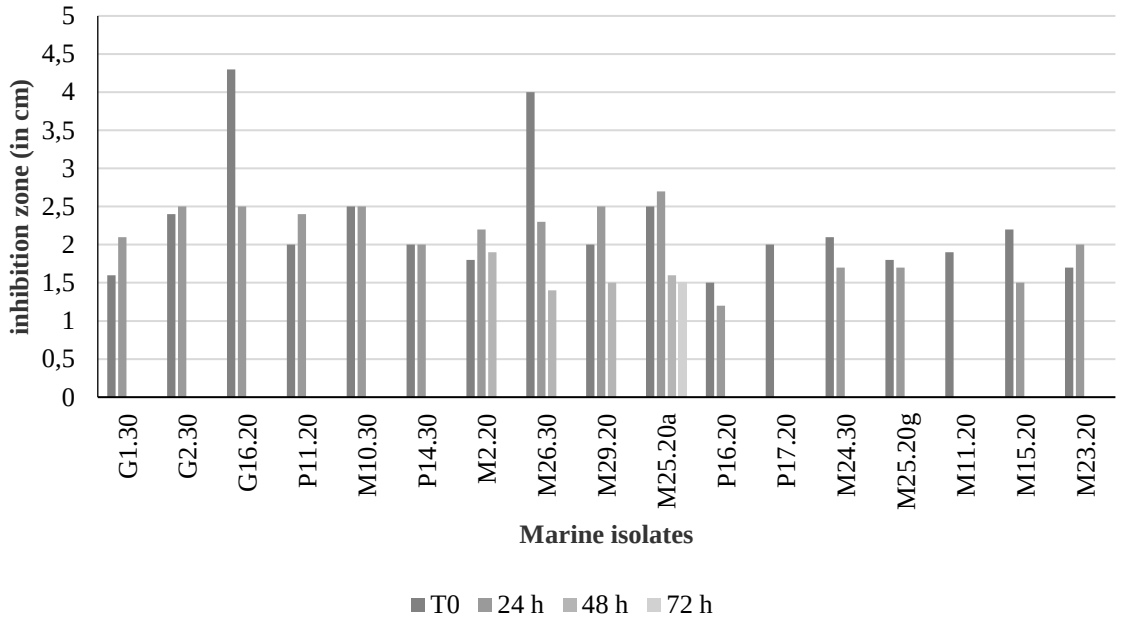


Figure 4.4 Kinetic production of antimicrobial compounds active against *P. damselae subp. piscicida* expressed as variation of inhibition zone (in cm).

Finally, well diffusion assay displayed (Figure 4.5 and Table 4.2) that *V. harveyi* was inhibited by CFS of 15 marine isolates with these trends: CFS of M24.30 and M25.20g marine isolates exhibited high antimicrobial activity only at T0 and 24; CFS of G1.30, G2.30, G16.20, M10.30, M2.20, M26.30, M29.20 and M25.20a marine isolates did not show antimicrobial activity at T0 but the production of antimicrobial compounds occurred at 24 h and CFSs of isolates P16.20, P17.20, M11.20, M15.20 and M23.20 showed antimicrobial activity only at T0 suggesting the production of antimicrobial compounds active against this indicator strains only in pre-cultures.

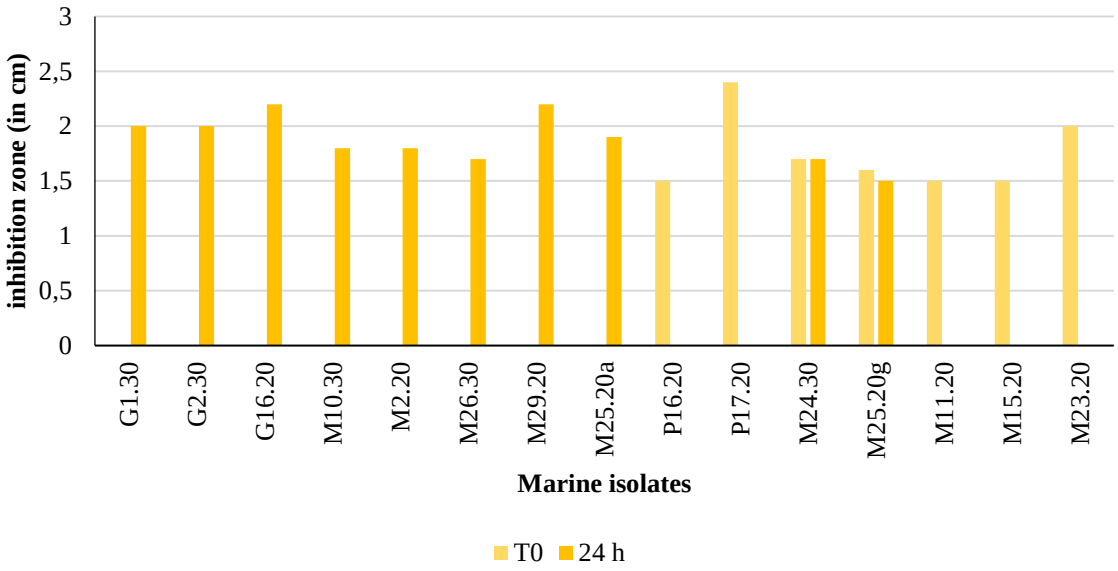


Figure 4.5 Kinetic production of antimicrobial compounds active against *V. harveyi* expressed as variation of inhibition zone (in cm).

Kinetics of growth and antimicrobial compounds production showed that all marine isolates were producers of one or more antimicrobial compounds and that growth and production kinetics of antimicrobial compounds were not linearly related.

As reported by Pérez (2014), often the optimal conditions for microbial growth not maximize the antimicrobial compounds production. In general, in fact, production of antimicrobial compounds such as bacteriocins depend of several factors (Abbasiliasi *et al.*, 2017) such medium pH and composition or growth conditions. Components such sodium chloride (NaCl) and surfactant in culture medium cause environmental stresses that maximize the bacteriocins production, stabilize their structure and avoid the complex formation (Iyapparaj *et al.*, 2013). pH plays key role in the control of enzymes that catalyses the post-translational processing of pre-bacteriocin to produce the active. In these cases, bacteriocins were classified as "pH-dependent primary metabolites" (Pérez *et al.*, 2014). It has also been observed that a reduced growth of many microorganisms in low pH cultures increases their ability to produce bacteriocins as there is a greater availability of ATP (energy) needed to activate the enzyme complexes. As well as medium composition and pH, the optical temperature for maximum bacteriocin production not be similar of optimal temperature for microorganism growth. Moreover, high temperatures associated with high pH values lower than the production of bacteriocins as the energy required to maintain the cellular homeostasis is high. The positive effect of variation of culture medium pH was observed for

7 marine isolates (G1.30, G16.20, M10.30, M2.20, M25.20g, M26.30 and P16.20) active against *Y. ruckeri*: an increase of the zone of inhibition were observed when 10% pre-inoculum (pH 8.2-8.8) was transferred in 200 mL of fresh mMSM (pH 8) (Figure 4.6). As reported by Hurst (1968) and Yang (2018), at low pH value the absorption of antimicrobial compounds (for example the bacteriocins) to cell walls decreases and so a high amount of these compounds is available in the culture medium.

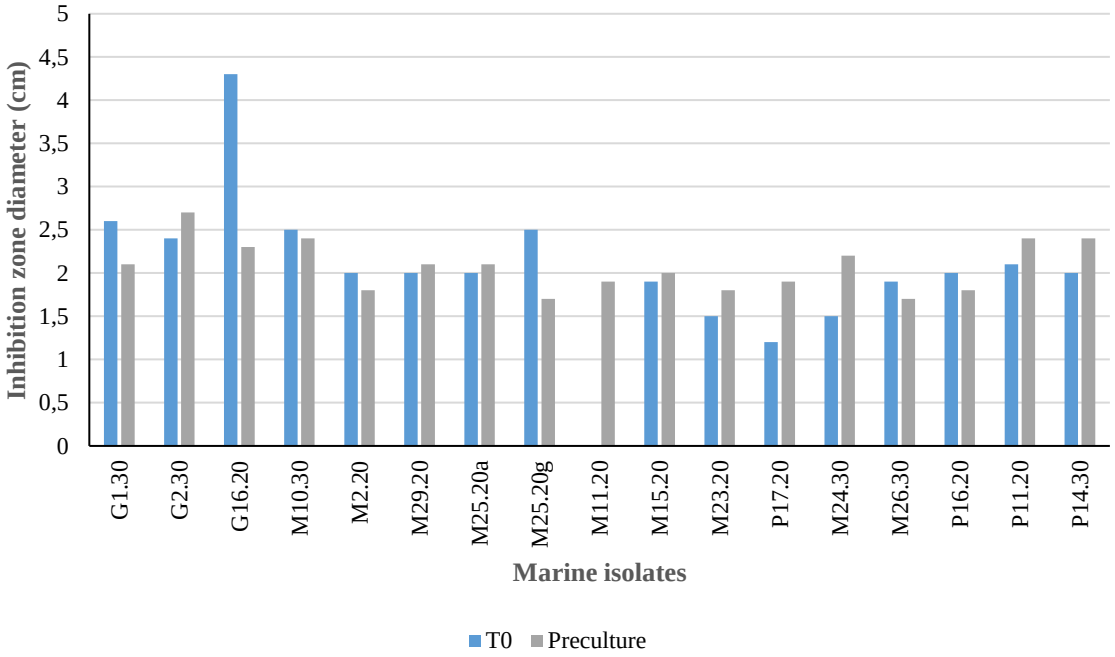


Figure 4.6 Comparison of the inhibition diameters of *Y. ruckeri* obtained at T0 and in preculture.

Similar trend was showed against *P. damselae* by 14 marine isolates (G2.30, G16.20, M10.30, M25.20a, M25.20g, M11.20, M15.20, M23.20, P17.20, M24.30, M26.30, P16.20, P11.20 and P14.30). 6 of these 14 marine isolates not showed inhibition zone in pre-inoculum but only a T0 (Figure 4.7).

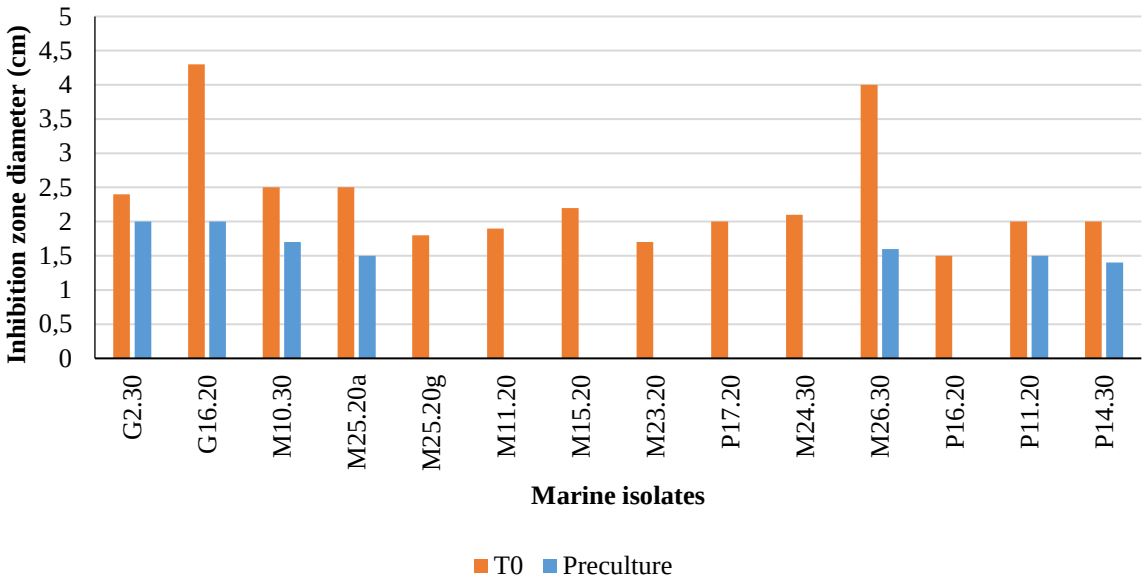


Figure 4.7 Comparison of the inhibition diameters of *P. damselae subp. piscicida* obtained at T0 and in the preculture.

Different tendency was observed against *V. harveyi*: 7 of 17 marine isolate (M25.20g, M11.20, M15.20, M23.20, P17.20, M24.30 and P16.20) release antimicrobial compounds after transfer of pre-inoculum in fresh mMSM and produced antimicrobial compounds in exponential phase (Figure 4.8).

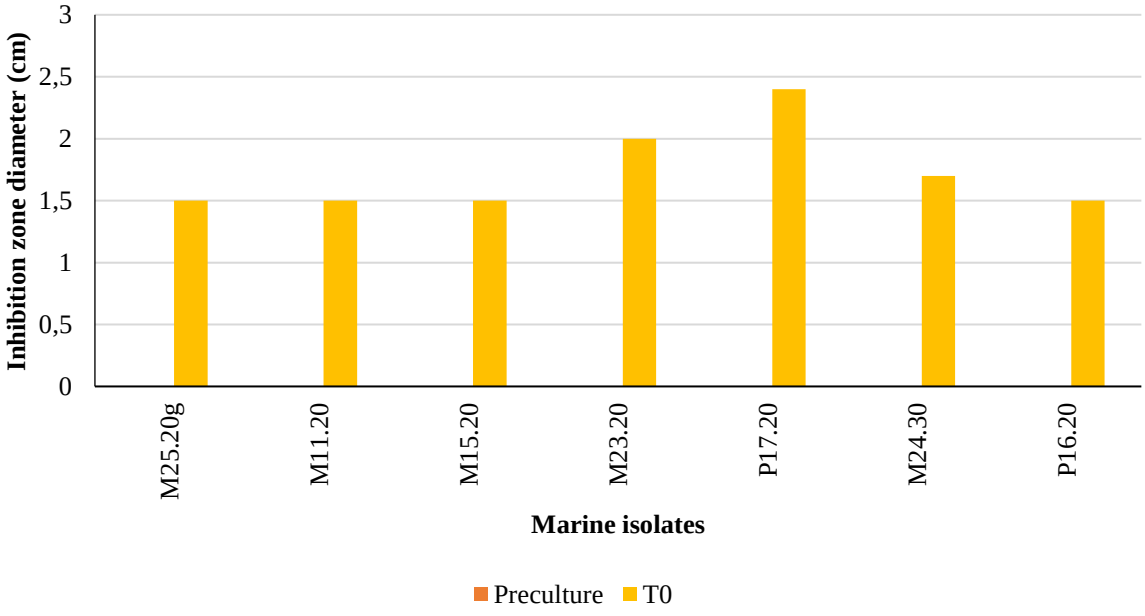


Figure 4.8 Comparison of the inhibition diameters of *V. harveyi* obtained at T0 and in the screening phase preculture.

Effects of pH, heat and proteolytic enzyme

The CFSs, collected after 24 h incubation, were used for evaluating the sensitivity of the antimicrobial compounds to the proteolytic enzyme proteinase K as well as to different extreme temperature (60, 80 and 120 °C) or pH (4 and 10) values. The results of these different treatments were compared to non-treated CFS (control) and expressed as residual antibacterial activity (%).

Bacteria exhibiting bacteriocin-like activities are currently being investigated as promising probiotics and antimicrobials for the seafood industry (Nguyen *et al.*, 2014; Offret *et al.*, 2019).

Table 4.3, 4.4 and 4.5 showed the effect of proteinase K on the antimicrobial activity of the tested CFS. With the exception of the CFS of isolates M24.30 and M10.30 which completely lost the activity towards *Y. ruckeri*, and CFS of isolate G16.20 which exhibited a reduced activity against *P. damselae;* the CFSs of all the remaining isolates showed a highly stable antimicrobial compounds retaining 100% of their antibacterial activity towards both of the indicator strains. The antibacterial activity of the CFS towards *V. harveyi* was not tested since at this sampling time, most of them did not exhibit inhibitory activity under control conditions (non treated CFS). The resistance of the antimicrobial compounds to protease K suggests that these compounds could be bacteriocins with low molecular weight i.e. peptides (Bacanli *et al.*, 2019; Ouzari *et al.*, 2008) or other metabolites that do not have a protein nature. Indeed, besides bacteriocins, other bacterial metabolites like for example EPS (Li *et al.*, 2014) and BS/BE could exhibit antimicrobial activity.

In our previous study (Raddadi *et al.*, 2017) the ability of most of the marine isolates studied here to produce biosurfactants stable to extremes of pH, temperature and water activity was found. The considered inhibition halo could therefore be the sum of the antimicrobial effects of bacteriocins and biosurfactants. Degradation effected by proteinase K would therefore be masked by the active antimicrobial permanence of biosurfactants.

Table 4.3 Residual activity (RA %) of the 17 CFSs active against *Y. ruckeri* after exposure to different pH/temperature and proteolytic enzyme.

Marine isolates	Treatments					
	pH 4	**pH 10**	**60 °C**	**80 °C**	**121 °C**	**Proteinase K**
M25.20g	93.33	86.67	93.33	93.33	93.33	100
M2.20	100.00	100.00	100.00	100.00	100.00	100
M24.30	80.00	95.00	100.00	100.00	100.00	0
M10.30	100.00	100.00	83.33	77.78	77.78	0
G16.20	100.00	100.00	100.00	100.00	100.00	100
M26.30	80.00	75.00	100.00	100.00	100.00	100
G1.30	90.48	90.48	100.00	100.00	85.71	100
G2.30	95.00	85.00	100.00	100.00	100.00	100
M29.20	100.00	88.89	94.44	94.44	94.44	100
P16.20	100.00	100.00	100.00	100.00	100.00	100
M15.20	87.50	93.75	93.75	93.75	93.75	100
P14.30	86.67	93.33	100.00	86.67	86.67	100
P11.20	100.00	88.89	88.89	88.89	77.78	100
M25.20a	94.74	94.74	100.00	89.47	89.47	87.5
M23.20	100.00	100.00	100.00	100.00	86.67	100
M11.20	92.86	92.86	100.00	100.00	100.00	100
P17.20	93.75	87.50	100.00	93.75	87.50	100

Table 4.4 Residual activity (RA %) of the 17 CFSs active against *P. damselae subp. piscicida* after exposure to different pH/temperature and proteolytic enzyme (NA: not applicable).

Marine isolates	Treatments					
	pH 4	**pH 10**	**60 °C**	**80 °C**	**121 °C**	**Proteinase K**
M25.20g	88.24	88.24	82.35	82.35	76.47	100
M2.20	90.91	100.00	86.36	81.82	81.82	100
M24.30	82.35	82.35	82.35	82.35	82.35	100
M10.30	80.00	88.00	80.00	80.00	72.00	91.67
G16.20	84.00	92.00	92.00	76.00	76.00	67.65
M26.30	100.00	100.00	100.00	100.00	100.00	100
G1.30	95.24	100.00	100.00	100.00	100.00	100
G2.30	100.00	92.00	80.00	80.00	80.00	100
M29.20	100.00	100.00	100.00	100.00	100.00	100
P16.20	100.00	100.00	100.00	100.00	100.00	100
M15.20	86.67	86.67	100.00	93.33	93.33	100
P14.30	100.00	100.00	100.00	100.00	100.00	100
P11.20	87.50	83.33	75.00	75.00	75.00	100
M25.20a	92.59	92.59	92.59	92.59	74.07	100
M23.20	90.00	90.00	90.00	90.00	80.00	100
M11.20	NA	NA	NA	NA	NA	NA
P17.20	NA	NA	NA	NA	NA	NA

Table 4.5 Residual activity (RA %) of the 17 CFSs active against *V. harveyi* after exposure to different pH/temperature and proteolytic enzyme (NA: not applicable).

Marine isolates	Treatments					
	pH 4	**pH 10**	**60 °C**	**80 °C**	**121 °C**	**Proteinase K**
M25.20g	100.00	100.00	100.00	100.00	93.33	100
M2.20	100.00	100.00	100.00	100.00	72.22	95
M24.30	88.24	94.12	100.00	94.12	88.24	NA
M10.30	88.89	88.89	77.78	77.78	77.78	100
G16.20	90.91	100.00	81.82	81.82	81.82	100
M26.30	100.00	100.00	100.00	100.00	100.00	99.90
G1.30	0.00	0.00	0.00	0.00	0.00	100
G2.30	85.00	100.00	85.00	85.00	80.00	100
M29.20	81.82	90.91	77.27	77.27	77.27	100
P16.20	NA	NA	NA	NA	NA	NA
M15.20	NA	NA	NA	NA	NA	NA
P14.30	NA	NA	NA	NA	NA	NA
P11.20	NA	NA	NA	NA	NA	NA
M25.20a	84.21	89.47	89.47	78.95	78.95	100
M23.20	NA	NA	NA	NA	NA	NA
M11.20	NA	NA	NA	NA	NA	NA
P17.20	NA	NA	NA	NA	NA	NA

4.5 Conclusion

A collection of 41 marine isolates was screened for the production of antimicrobial compounds exhibiting activity against farmed fish pathogens (*Y. ruckeri*, *V. harveyi*, *P. damselae* and *F. psychrophilum*). Among them, 4 isolates were producers of one or more molecules active against three fish pathogens and 13 inhibited the growth of both *Y. ruckeri* and *P. damselae*. Furthermore, were selected 8 marine bacterial isolates able to produce stable compounds (pH/temperature extremes and proteinase K treatment) active against 3 fish bacterial pathogens.

Further studies should be dedicated to the recovery of these active compounds, the elucidation of their nature and the evaluation of their effectiveness in inhibiting fish pathogens in rearing conditions in order to use them as friendly alternatives to antibiotics for a more sustainable aquaculture sector.

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5. GENERAL CONCLUSIONS

The contribution of aquaculture to global food production in the fishing industry is expanding. The sustainability of the sector urge aquaculture industry to develop processes aimed at: i) improving quantity and quality of fish, ii) containing costs, iii) reducing environmental threats by minimizing wastes and valorizing by-products (Jennings *et al.*, 2016; EU, 2019).

To date, several studies were carried out on the valorisation of fishing industry by-products/wastes and many of the products obtained are available in commerce (Zhang *et al.*, 2019). However, only very few reports are available on the selection/application of probiotics in aquaculture as well as on the use of antimicrobial compounds from microbial origin for the control of bacterial pathogens. In this context, the aim of the research activity is to select novel safe marine bacteria able to produce LC-PUFAs and/or antimicrobial compounds active against aquaculture fish pathogenic bacteria with the perspective of their application in aquaculture.

In this context the aim of the research activity is hence to apply biotechnological approaches to research new antimicrobial compounds and EPA and DHA sources for the sustainability of aquaculture systems.

The first part of the work has been dedicated to the selection of LC-PUFAs bacterial producers. For this purpose, 209 marine bacterial isolates from the Mediterranean Sea was screened in order to select new LC-PUFAs, EPA and DHA bacterial producers. After colourimetric screening, ITS typing, GC-FID analysis and molecular identification of marine isolates, different *Marinobacter* sp., *Thalassospira* sp and *Halomonas* sp. isolates could produce DHA. The maximum achieved productivity of DHA by *Marinobacter* sp G16.20 was 1.85±0.371 mg/g. Production of LC-PUFAs from these genera has not been reported previously, to the best of our knowledge. All isolates from this study produced relatively low levels of DHA compared with levels produced by strains in other genera such as *Colwellia*. Nevertheless, considering that bacteria from the genus *Marinobacter* have never been reported to be involved in human or animal pathology, strain *Marinobacter* sp G16.20 has the potential to be used as an LC-PUFAs source ingredient for the preparation of feed in aquaculture sector, after production process optimization.

In the second part of the work, efforts has been concentrated on the selection of antimicrobial compounds producers and the characterization of the stability of the produced compounds. For this purpose, the 15 isolates having the ability to produce DHA together with other 26 marine isolates from collection was screened for the production of antimicrobial compounds exhibiting activity against farmed fish pathogens (*Y. ruckeri*, *V. harveyi*, *P. damselae* and *F. psychrophilum*). After screening and preliminary characterization (sensitivity to pH, temperature and proteinase K), were selected 8 marine bacterial isolates able to produce stable compounds (pH/temperature extremes and proteinase K treatment) active against *Y. ruckeri*, *V. harveyi*, *P. damselae*. Further studies should be dedicated to the recovery of these active compounds, the elucidation of their nature and the evaluation of their effectiveness in inhibiting fish pathogens in rearing conditions in order to use them as friendly alternatives to antibiotics for a more sustainable aquaculture sector.

In conclusion, among a large collection of marine bacterial isolates screened, 10 strains able to produce both antimicrobial compounds active against different aquaculture fish pathogenic bacteria and LC-PUFAs were selected. These results are interesting and promising since these isolates belong to bacterial species that have never been reported to be involved in human or animal pathology and exhibited traits that give them high potential for the application in aquaculture in order to optimize fish production.