

POTENTIAL IMPACT OF EMERGING BACTERIAL PATHOGENS ON SHELLFISH PRODUCTION

Faiz Ur Rahman

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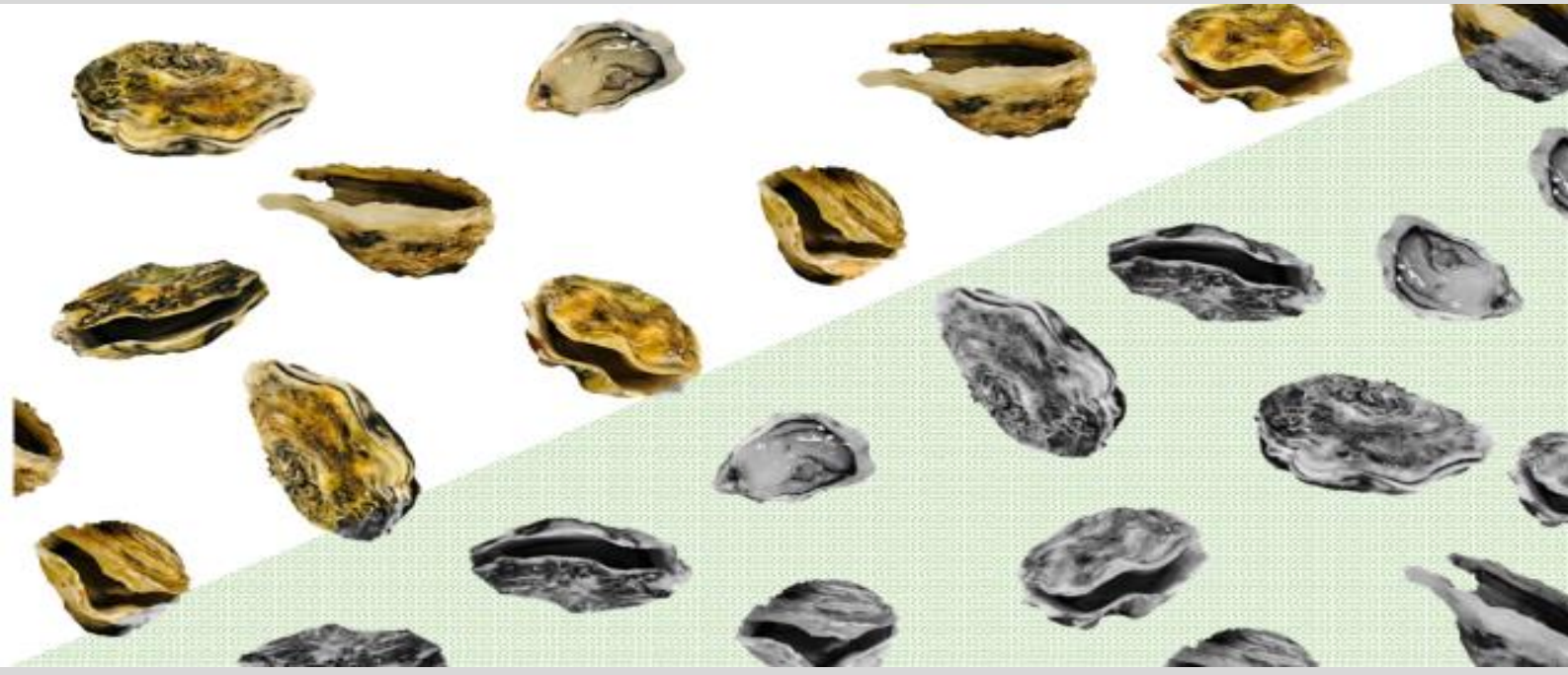
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UNIVERSITAT ROVIRA i VIRGILI

IRTA RESEARCH & TECHNOLOGY FOOD & AGRICULTURE

Potential impact of emerging bacterial pathogens on shellfish production.

FAIZ UR RAHMAN



DOCTORAL THESIS
2023

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POTENTIAL IMPACT OF EMERGING BACTERIAL PATHOGENS ON SHELLFISH PRODUCTION.

DOCTORAL THESIS 2023

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To my beloved father and mother.

The love and sacrifice that you gave for me so I can study.

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SUMMARY

SUMMARY

SUMMARY

Arcobacter-like species are rod-shaped gram-negative bacteria found ubiquitously in nature. They are present in food and dairy products, chicken, pigs, turkeys, fish, and shellfish etc. They are also present in fresh, brackish, and marine water. Many species of the *Arcobacter* are considered zoonotic pathogens, while many have been suspected to have potential of causing diseases in their hosts. In the terrestrial environment many species have been found associated with the diseases of animals and humans while some species recovered from the shellfish and marine environment have the potential to cause diseases in humans when they consume raw seafood. Although these bacteria are regularly found in shellfish however, their role in shellfish production has not been studied extensively. Thus, the aim of the present thesis was to study the role of *Arcobacter*-like species in shellfish production. Consequently, a sequential approach was followed starting by isolating *Arcobacter*-like spp. from the shellfish growing in the Ebro Bays production zones, specifically Pacific oyster (*Crassostrea gigas*) during mortality and non-mortality episodes. Since *Arcobacter*-like spp. require an enrichment step prior its isolation on solid media, and since sodium chloride (NaCl) favors the recovery of halophilic strains, a total of 24 formulations of enrichment broth were designed, aiming to improve further the recovery of marine *Arcobacter* species. Consequently, it was found that the growth of *Arcobacter*-like spp. from shellfish (*C. gigas*) was significantly higher in Arcobacter-CAT-broth when it was supplemented with 50% artificial seawater (ASW), therefore, this enrichment broth was used for isolating and culturing of *Arcobacter*-like bacteria in further studies. The next phase, consisted of the epidemiological study of the mortality episodes of *C. gigas* from the Ebro Delta, shellfish growing zones (Spain). This was carried out for the known pathogens in the area, such Ostreid Herpes virus 1 (OsHV-1), *Vibrio aestuarianus*, *V. splendidus* clade and, additionally, this study incorporated the screening for *Arcobacter*-like spp., intending to assess its role in such episodes. It was found that OsHV-1 and *V. aestuarianus*

were found in *C. gigas* only in mortality episodes samples, while *V. splendidus* clade and *Arcobacter*-like spp. were present in both mortality and non-mortality episodes. As, *Arcobacter*-like bacteria consists of many different species, the further genetic profiling and phylogenetic analysis of the isolates revealed that mainly *Malaciobacter marinus* was present in the samples from mortality episodes, while it was not isolated from samples of non-mortality episodes. An *M. marinus* specific conventional PCR and qPCR assays were developed using *M. marinus* specific Mm-rpoB primer set. Taking this finding into account, the role of *M. marinus,* as a potential pathogen for *C. gigas,* was studied by *in-vivo* bacterial challenge experiments in *C. gigas.* Results indicated that the mortality in *C. gigas* was 15% higher when both *V. aestuarianus* (a known pathogen of *C. gigas*) and *M. marinus* were experimentally infecting the animal, than when infection was set only with *V. aestuarianus,* indicating a potential synergistic role of *M. marinus* in infection in *C. gigas.* Furthermore, the interaction of two *M. marinus* isolates from mortality episode with the hemocytes of Mediterranean mussel (*Mytilus galloprovincialis*) and *C. gigas* was studied. It was found that *M. marinus* was moderately pathogenic to early larvae of *M. galloprovincialis* while in adult the lysosomal membrane stability (LMS) was decreased in *M. galloprovincialis* and *C. gigas* hemocytes indicating stress given to the host. However, reactive oxygen species (ROS) and lysozyme were released as defense response to the bacteria in the host. These results indicated that, although *M. marinus* is not a potent pathogen for the shellfish tested by itself, it might have the potential to increase the severity of mortality episodes when co-infecting with other bacterial pathogens, such as *V. aestuarianus*, under a polymicrobial infection scenario.

RESUMEN

Las especies similares a *Arcobacter* son bacterias gram-negativas en forma de bastón que se encuentran de forma ubicua en la naturaleza. Están presentes en alimentos y productos lácteos, pollo, cerdos, pavos, pescados y mariscos, etc. También están presentes en agua dulce, salobre y marina. Muchas especies de *Arcobacter* se consideran patógenos zoonóticos, mientras que se sospecha que muchas tienen el potencial de causar enfermedades en sus huéspedes. En el ambiente terrestre se han encontrado muchas especies asociadas con enfermedades de animales y humanos mientras que algunas especies recuperadas del marisco y ambiente marino tienen el potencial de causar enfermedades en humanos cuando consumen mariscos crudos. Aunque estas bacterias se encuentran regularmente en los mariscos, su papel en la producción de mariscos no se ha estudiado exhaustivamente. Por lo tanto, el objetivo de la presente tesis fue estudiar el papel de las especies similares a *Arcobacter* en la producción de mariscos. En consecuencia, se siguió un enfoque secuencial comenzando por aislar *Arcobacter*-like spp. de los moluscos que crecen en las zonas de producción de las Bahías del Ebro, en concreto de la ostra del Pacífico (*Crassostrea gigas*) durante los episodios de mortalidad y no mortalidad. Dado que *Arcobacter*-like spp. requieren un paso de enriquecimiento previo a su aislamiento en medios sólidos, y dado que el cloruro de sodio (NaCl) favorece la recuperación de cepas halófilas, se diseñaron un total de 24 formulaciones de caldo de enriquecimiento, con el objetivo de mejorar aún más la recuperación de especies marinas de *Arcobacter*. En consecuencia, se encontró que el crecimiento de *Arcobacter*-like spp. de mariscos (*C. gigas*) fue significativamente mayor en el caldo Arcobacter-CAT cuando se complementó con un 50% de agua de mar artificial (ASW), por lo tanto, este caldo de enriquecimiento se usó para aislar y cultivar bacterias similares a *Arcobacter* en estudios posteriores. La siguiente fase, consistió en el estudio epidemiológico de los episodios de mortalidad de *C. gigas* del Delta del Ebro, zonas de cultivo de moluscos (España). Este se realizó para los patógenos conocidos en el área,

tales como Ostreid Herpes virus 1 (OsHV-1), *Vibrio aestuarianus*, *V. splendidus* clade y, adicionalmente, este estudio incorporó el tamizaje para *Arcobacter*-like spp., con la intención de evaluar su papel en tales episodios. Se encontró que OsHV-1 y *V. aestuarianus* se encontraron en *C. gigas* solo en muestras de episodios de mortalidad, mientras que el clado *V. splendidus* y *Arcobacter*-like spp. estuvieron presentes tanto en episodios de mortalidad como de no mortalidad. Dado que las bacterias similares a *Arcobacter* se componen de muchas especies diferentes, el perfil genético y el análisis filogenético-posteriores de los aislamientos revelaron que principalmente *Malaciobacter marinus* estaba presente en las muestras de episodios de mortalidad, mientras que no se aisló de muestras de episodios de no mortalidad. Se desarrollaron ensayos de qPCR y PCR convencionales específicos para *M. marinus* utilizando un conjunto de cebadores Mm-rpoB específicos para *M. marinus*. Teniendo en cuenta este hallazgo, se estudió el papel de *M. marinus*, como patógeno potencial de *C. gigas*, mediante experimentos de desafío bacteriano *in vivo* en *C. gigas*. Los resultados indicaron que la mortalidad en *C. gigas* fue un 15 % mayor cuando *V. aestuarianus* (un patógeno conocido de *C. gigas*) y *M. marinus* infectaron experimentalmente al animal, que cuando la infección se estableció solo con *V. aestuarianus*, lo que indica un papel sinérgico potencial de *M. marinus* en la infección en *C. gigas*. Además, se estudió la interacción de dos aislamientos de *M. marinus* del episodio de mortalidad con los hemocitos de mejillón mediterráneo (*Mytilus galloprovincialis*) y *C. gigas*. Se encontró que *M. marinus* era moderadamente patógeno para las larvas tempranas de *M. galloprovincialis*, mientras que en adultos la estabilidad de la membrana lisosomal (LMS) disminuyó en los hemocitos de *M. galloprovincialis* y *C. gigas*, lo que indica estrés en el huésped. Sin embargo, se liberaron especies reactivas de oxígeno (ROS) y lisozima como respuesta de defensa a las bacterias en el huésped. Estos resultados indicaron que, aunque *M. marinus* no es un patógeno potente para los mariscos analizados por sí mismo, podría tener el potencial de aumentar la gravedad de los episodios de mortalidad cuando se

coinfecta con otros patógenos bacterianos, como *V. aestuarianus*, bajo un escenario de infección polimicrobiana.

1. INTRODUCTION

INTRODUCTION

Introduction

Shellfish production is one of the main aquaculture practices worldwide, cultivated as a cash crop and a good source of quality protein (Sapkota et al., 2008). About 10-12% of the world population is dependent for their income on fisheries and aquaculture (Solomieu et al., 2015; Lam, 2016). The European Union is the 5th largest producer (3.3%) of global fisheries and aquaculture production worldwide (EC, 2020). Aquaculture has been recognized as a potential solution to, as well as a contributor to, the global reduction in fishery stocks several decades ago (Naylor et al., 2000). Aquaculture production grew worldwide from 16 million tons to 115 million tons between 1988 and 2018 comprising of about 430 different farmed species ranging from seaweed, algae, to fish and shellfish (FAO 2019; Cai et al., 2022). The technical development and continuous growth of fish aquaculture and expanded culture of bivalves and seaweeds, with potential to provide a wide range of food, industrial and ecosystem services, shows that land and sea are becoming increasingly entangled (Naylor et al., 2021). Through expansion in aquaculture, consumers in low to high-income countries have profited from year-round availability and access to aquatic foods, which are high in protein and micronutrients (Bene et al., 2016).

1.1 Global Aquaculture Production

Global aquaculture production has gradually increased from 16 Mt in 1988 to 115 Mt in 2018, more than seven-times, in live-weight volume (FAO 2019; Cai et al., 2022). Seaweeds, carps, bivalves, tilapia, and catfish were the primary species categories that contributed to the top 75% of aquaculture production in 2018 (Naylor et al., 2021). Although the output of marine fish species, as well as crustaceans, grew fast during this time, it was overshadowed by the live-weight volume of marine bivalves and seaweeds, as well as freshwater aquaculture production (Edwards et al., 2019).

INTRODUCTION

Asia continues to be the world's greatest aquaculture producer, with 92 percent of live-weight volume of animals and seaweeds production, adopting high number of techniques for culturing (FAO 2019). In practically every aspect of aquaculture production, China plays a major role. Since 2000, the country has been the world's top producer, processor, and trader of fish, crustaceans, and mollusks, and has also emerged as a major consumer due to the fast growth of the industry (Naylor et al., 2021). Europe produced about 4% of the world's aquaculture production in 2018, with an output of about 3.1 million tons (Rocha et al., 2022). Marine species make up most of the European aquaculture, accounting, by 2018, for about 83.5% of the continent's total production (FAO, 2020; FEAP, 2020), thus leaving most European aquaculture to be carried out in marine and coastal environments. Fish and mollusks were the main groups reared, representing nearly the whole of the aquaculture production in Europe in 2018; the production of crustaceans and other aquatic animals represented a minor slice of Europe's production. Norway is the leading European country in aquaculture production, with an output of more than 50% of the total production in Europe by volume in 2018 (about 1.4 million tons of live weight) and the seventh main aquaculture producer worldwide (EUMOFA 2021; Rocha et al., 2022). Its production focuses mainly on the rearing of Atlantic salmon, which has been highly optimized to allow maximum production, adapting to rather rough conditions (Bjelland et al., 2015). Spain, the United Kingdom, and France were the following top European producers accounting together for about 22.3% of the total European production in 2018 (EUMOFA 2021). Southern European countries i.e., Spain, Italy, Greece, Turkey, and Portugal have a long history in aquaculture production and are responsible for the share of production of marine finfish, some freshwater fish, and mollusks which take the largest share of the production (FAO 2020). Spain, France, and Italy, although producing several commercially important marine, diadromous and freshwater finfish species, stand out from the rest of the European

producing countries due to the highest share of each nation's total aquaculture production being mollusk farming (Botta et al., 2020; APROMAR, 2021; Rocha et al., 2022). Bivalve mollusks (mussels, oysters, and clams) are dominant in Spain, France, and Italy (European Parliament 2022). Spain produced a total of 307,168 tons of aquaculture in 2020, having a major share of mussels (233,467 t), sea bass (21,709 t) and rainbow trout (19,400 t) as main species (APROMAR, 2021).

1.1.1 *Crassostrea gigas* Production

Mollusks are an important phylum of invertebrate animals, and very important species for aquaculture, with a total share of 17.7 million tons production globally in 2018 and increasing (FAO 2020). One of the main species of bivalve mollusks, the Pacific oyster *Crassostrea gigas* was introduced from Asia to various countries all over the world (e.g., Argentina, Australia, Brazil, Canada, Chile, Mexico, New Zealand, Namibia, South Africa, USA, and various European countries, including Spain) during the 20th century, due to its rapid growth and reliance adapting to changing environmental conditions and has been prioritized for cultivation over other similar species (Ruesink et al, 2005; Prado-Alvarez et al., 2016; De Lorgeril et al., 2018; Song et al., 2019). Consequently, *C. gigas* is the main oyster species farmed worldwide and represents more than 98% of oyster production (Guo 2009; Azema et al., 2017; Petton et al., 2021). Worldwide production of *C. gigas* was 643.5 thousand tons in 2018 with a total share of 3.7% of the total mollusk's production (FAO 2020). About 10-12% of the world's population depends for their income on fisheries and aquaculture (Solomieu et al., 2015). China is, by far, the leading producer followed by South Korea, Japan, and France (Petton et al., 2021). European Union production of the *C. gigas* was 100,187 with a value of 401.75 million euros in 2019 (APROMAR 2021). France is the top producer of Pacific oyster in the EU, with 45% of the total aquaculture production in 2019 (EUMOFA, 2020). Shellfish production is the main aquaculture sector in

Spain, led by the mussel aquaculture in Galicia (FAO 2022). Approximately 494 tons of Pacific oyster *C. gigas* were produced in Spain in 2020, mainly in Galicia, Catalonia, Andalusia, Asturias, Cantabria, and the Valencian Community having a total value of 4.0 million euros (APROMAR, 2021). The Ebro delta in Catalonia (Northeast Spain) is the largest *C. gigas* producing area on the Mediterranean coast of Spain, and the second largest shellfish producing region in the country.

1.2 Shellfish Diseases

Infectious diseases affecting cultured bivalves are continuously affecting the farms since the start of the commercial production of shellfish mostly caused by different microorganisms (Destoumieux-Garzón et al., 2020; Diwan et al., 2022). Although hatcheries are the most controllable part of the system in mollusk aquaculture, infectious organisms can enter a marine shellfish hatchery from three main sources: brood stock, saltwater supply, and algal food source (Elston et al., 2008). Once an infectious organism has established itself in the system, it may be able to survive without being introduced again, generating permanent or recurrent losses (Smith et al., 2003; C De et al., 2014). In clam hatcheries, bacterial infections are the most prevalent illness. Infections with viruses have also been observed in farms, and these are usually passed down through contaminated brood stock (Oliveira et al., 2011). In both larval and juvenile bivalves, *Vibrio* infections are highly aggressive and spread quickly where larvae have a limited ability to recover from infection (Canesi and Pruzzo, 2016). Invasive infections use the pallial tissues (mantle and gill) as a route of entry to initiate the infection while the digestive tract shows barrier to the infection (Allam et al., 2013). Infections of ligament are one of the most frequent and widespread illnesses among juvenile shellfish, caused by gliding bacteria (McGladdery, 2011). The bivalves with subpallial bacterial infections develop mantle necrosis and then bacteria enter coelomic cavity (Elston et al., 2008). The nature of infectious illnesses differs depending on the species of pathogens involved in the process (Beaz-Hidalgo

et al., 2010). In adult oysters, vibrios are a prevalent etiologic agent, whereas gliding bacteria harm several clam species (Elston and Ford, 2011; Cantrell et al., 2020). Infections of larval and juvenile shellfish with virus infections, like iridovirus and herpes virus, have been found in larval and seed of bivalves from numerous continents (Bandín and Dopazo, 2011; Pernet et al., 2016; He et al., 2019; Hassou et al., 2020). Contaminated brood stock is the cause of the several diseases, therefore, management implementing cleanliness techniques in closed commercial hatcheries systems, can significantly reduce the transmission of infections (Oliveira et al., 2011; Dubert et al., 2017). Opportunistic parasitic ciliates like *Marteilia* and *Perkinsus*, can also infect immature shellfish like *C. gigas* through the mantle tissue, causing a long-term illness (Hine and Thorne 2000; Ben-Horin et al., 2015; Paladini et al., 2017). Therefore, disease control in hatcheries needs a well-structured disease management plan, based on risk, which would prevent the entry of the main pathogens prevalent in the zone, and avoid new ones coming from other production areas. In extensive cultures, with no barriers, avoiding the entry and movements of infected stock and materials, equipment and tools is paramount (Arzul et al., 2021).

1.2.1 *Crassostrea gigas* Bacterial Diseases

Bacteria diseases are one of the most severe causes of mortalities in aquaculture systems, owing to their direct transfer and capacity to spread quickly in the crowded settings that are common in culture.

1.2.1.1 *Vibrio* Diseases

The increase in sea surface temperature, due to global climate change, has a negative impact on the environment. The increase in *Vibrio* related infections in marine organisms is one of the consequences of that negative impact (Ushijima et al., 2018). Vibrios are very diverse heterotrophic bacteria having various metabolically and genetically distinct groups, normally

occurring in marine environments from surface to deep water and coastal to open ocean environments while some occur even in freshwater and brackish water environments (Thompson et al., 2004a; Thompson and Polz, 2006). Several *Vibrio* species are important because the infections that they produce can impact the economic and public health as they are potentially pathogens to humans and animals (Thompson et al., 2004b, 2005; Stewart et al., 2008). Vibrios are genetically, ecologically, and evolutionary one of the most extensively studied bacterial models in wild environment (Takemura et al., 2014).

Vibrio species can be found in both planktonic and animal-associated microbial ecosystems in the ocean (Le Roux et al., 2016; Foulon et al., 2016; Lopez-Joven et al., 2018; Rubiolo et al., 2019; Diner et al., 2021; Priya et al., 2022). They are Gram-negative bacteria that belong to the Proteobacteria phylum and the Vibrionaceae family. *Vibrio* species and strains have been shown to be harmful for fish and shellfish in increasing numbers, making them a cause of worry in aquaculture (Saulnier et al., 2010). Non-pathogenic *Vibrio* species include *V. coralliilyticus*, *V. splendidus*, *V. crassostreae*, and *V. tasmaniensis* and are commensal bacteria found in healthy oysters that can become opportunistic when their health is harmed by illness or stressful events like temperature changes (Vezzulli et al., 2010; Lasa et al., 2019). It is often unclear whether the *Vibrio* species isolated from infected oysters are the causal agent, the secondary opportunistic infections, or mainly commensals bacteria (Le Roux et al., 2016). After demonstrating significant levels of *Vibrio* spp. in the hemolymph of moribund oysters, the significance of these bacteria in the summer mortality episodes of oysters was considered important (Lokmer et al., 2015). Several pathogenic *Vibrio* species have been isolated during Pacific oyster mass mortality outbreaks, both in the presence or absence of OsHV-1 (Garnier et al., 2007; Sauvage et al., 2009; De Decker et al., 2011; 2013; Keeling et al., 2014; King et al., 2019c; 2020). *Vibrio splendidus* (Lacoste et al., 2001a; 2001b; Le Roux et al., 2002; Pernet et al., 2012), *V. aestuarianus* (Garnier et al., 2008; Saulnier et al., 2009), *V. harveyi* (Garnier et

al., 2008; Saulnier et al., 2009; Go et al., 2017; Green et al., 2019; Nordio et al., 2021), *V. tubiashii*, *V. coralliilyticus* (Richards et al., 2015; Kim et al., 2020; Madison et al., 2022) and *V. alginolyticus* (Yang et al., 2021) were among the isolates whose virulence was established.

Several pathogens can be found simultaneously in an infected host during disease outbreak of Pacific oyster. In a longitudinal investigation conducted during the 2010-2011 summer mortality outbreak linked with OsHV-1 infection of *C. gigas* in New Zealand, both *V. splendidus* and *V. aestuarianus* were isolated (Keeling et al., 2014). Furthermore, Gay et al. (2004) demonstrated, in an experimental setting, the pathogenicity of two *Vibrio* strains from the *Splendidus* super-clade obtained from a summer mortality episode. A new *Vibrio* species (*Vibrio crassostreae*) was recently discovered in the hemolymph of sick oysters (Bruto et al., 2017). This species was thought to be a harmless oyster colonizer that became virulent after gaining a virulence plasmid (Bruto et al., 2017). Furthermore, *V. tubiashii*, a pathogen identified from North American shellfish hatcheries, has been linked to illness in *C. gigas* larvae and juveniles (Elston et al., 2008; Estes et al., 2004). In 2005, *V. coralliilyticus* resurfaced in the United States, causing massive losses in Pacific oyster larvae and juveniles (Elston et al., 2008). Pacific oysters are thought to be colonized by a heterogeneous array of vibrios in most cases (Le Roux et al., 2016; Petton et al., 2015b). These persistent vibrios in infected oysters can be mutualistic, opportunistic, or pathogenic (Lemire et al., 2015).

In pathogenic *Vibrio* strains of Pacific oysters, metalloprotease is a virulence factor (Saulnier et al., 2010). Injection into *C. gigas* of *Vibrio* extracellular products has been proven to be an important factor of fatality (Le Roux et al., 2007). *Vibrio* spp. isolates have been tested for virulence using metalloprotease-like activity in culture supernatant fluid as a laboratory test (Saulnier et al., 2010). Some pathogenic strains of *V. splendidus*, *V. aestuarianus*, and *V. tubiashii* have been shown to have metalloproteases (Labreuche et al., 2006; Le Roux et al., 2007; Saulnier et al., 2010).

In a mortality episode of Pacific oysters, Petton et al. (2015b) showed the fast colonization of several vibrios prior to OsHV-1 replication. Furthermore, it has been demonstrated that a high load of OsHV-1 alone was inadequate to trigger the complete manifestation of the disease, indicating that bacteria may play a part in the development of disease (Petton et al., 2015b; Pathirana 2020; King et al., 2019c). The addition of broad-spectrum antibiotic chloramphenicol to the oyster tanks every two days was enough to suppress the growth of microbiota, including *Vibrio*, in the oyster tissues, which resulted in a lower mortality following exposure to OsHV-1 than oysters that were not treated (Petton et al., 2015b). De Lorgeril et al. (2018) recently looked at the complicated etiology of OsHV-1-related sickness indication that an immune-compromised condition was caused by an initial infection with the virus in juvenile oyster hemocytes, which was followed by bacteremia with opportunistic bacteria. Histological findings indicated bacterial accumulation and invasion of hemocytes both inside and outside the gill tissues of oysters infected with OsHV-1, indicating that secondary bacterial involvement was required to complete the disease manifestation (De Lorgeril et al., 2018). Lasa et al. (2019) used a molecular technique, the 16S rRNA next generation sequencing, to detect microbial communities associated with *C. gigas* samples in microbiota collected during mortality events at different European locations, to support the increasing concept of a polymicrobial etiology in Pacific oyster mortalities. The study discovered symptoms of a disrupted microbiota as well as the presence of previously unreported, potentially dangerous bacteria, particularly from the *Vibrio* and *Arcobacter* genera (Lasa et al., 2019). It has been described that the Pacific oyster microbiome is affected by artificial seawater conditions, such as those used in oyster depuration (Pathirana, 2020). Clean seawater is commonly used in the depuration process, which has been treated in a series of stages to eliminate pollutants, including sand filtering, UV treatment, ozonation (infusion of ozone into water), and biofiltration (Vezzulli et al., 2018). Depuration is intended to remove harmful microorganisms

from the circulating water where oysters are cultured, however, treatment hasn't always been successful in eradicating naturally existing marine vibrios like *V. parahaemolyticus* and *V. vulnificus* that are pathogenic to humans (Lee et al., 2008) and *V. aestuarianus* and *V. splendidus* clade that are pathogenic to oysters (Lacoste et al., 2001a; Vezzulli et al., 2018). Vezzulli et al. (2018), on the other hand, found an increase in the *Vibrio* percentage in the hemolymph of *C. gigas* after depuration. The persistence of *Vibrio* during depuration was interpreted by Vezzulli et al. (2018) as a characteristic of this microbe derived possibly from the long co-evolutionary history with their invertebrate hosts and that corresponded to a permanent resident microbiota able to increase in abundance under changing environmental conditions. Similarly, other studies found that the amount of *Vibrio* in *C. gigas* tissues was also high in the moribund oysters translocated to a different location from its host environment (Lokmer et al., 2016a; 2016b).

Le Roux et al. (2016) argue that the pathophysiology of oyster sickness includes a coevolutionary interplay between vibrios, oysters, and their microbiome, further strengthening the idea of Vibrio's co-evolutionary involvement. In this respect, *Vibrio* spp. can play a secondary opportunistic function (De Lorgeril et al., 2018). The rise of the sea surface temperature plays an important role in driving the pathogenic cycle of bacteria in the host, in fact it has been observed that the virulence potential of *V. coralliilyticus* strains increased due to rise in water temperature producing coral bleaching and mortality of Pacific oysters at larval stages (Ushijima et al., 2018). The recent discovery of *V. coralliilyticus* strain BAA-450 as a new pathogen for Pacific oyster larva increased the importance to study the virulence potential of this *Vibrio* spp. (Richards et al., 2015). The U.S. shellfish hatcheries suffered potential loss due to this new shellfish pathogen which caused several sporadic outbreaks along the U.S. coast in early 2000 with up to 80% mortality in the larva (Estes et al., 2004; Elston et al., 2008; Richards et al., 2015). Cross-species virulence is possible by certain pathogens found in the sharing environment as it was observed in *V. coralliilyticus*. In this species the virulence factors

i.e., the regulatory protein ToxR and the regulated protein OmpU, both were responsible for cross species infections in coral and Pacific oyster larva (Ushijima et al., 2018). The ToxR is also known for its positive regulation of an important outer membrane protein OmpU, responsible for adherence in several other vibrio pathogens like *V. vulnificus* and *V. cholera* to human cells and in *V. tasmaniensis* to oyster hemocytes (Sperandio et al., 1995; Provenzano and Klose et al., 2000; Goo et al., 2006; Duperthuy et al., 2011). The target organisms for *V. coralliilyticus* are beyond Pacific oysters and the susceptible hosts include several other shellfish species such as Kumamoto oyster (*Crassostrea sikamea*), Eastern oyster (*Crassostrea virginica*), Green shell mussel (*Perna canaliculus*), and Geoduck clam (*Panopea generosa*) (Estes et al., 2004; Elston et al., 2008; Kesarcodi-Watson et al., 2009; Richards et al., 2015). It is not clear whether *V. coralliilyticus* strains contains different virulence factors for different hosts or possess a single set of virulence factors responsible for triggering disease in various hosts, but the infections of Pacific oyster larvae by coral-associated pathogenic strains and a comparable temperature response indicate that virulence factors are shared (Ushijima et al., 2018).

1.3 Emerging Bacterial Pathogens of *Crassostrea gigas*

As commented, the Pacific oyster, *C. gigas*, is widely cultivated, even though disease outbreaks have been progressively affecting its production (Azema et al., 2015). Although viral and bacterial are the most common etiological agents of these outbreaks (Friedman et al., 2005; Garnier et al., 2007; Segarra et al., 2010; King et al., 2019b), there are instances when no obvious etiological agent can be identified (Go et al., 2017; King et al., 2019a). The Ostreid herpes virus 1 (OsHV-1) and its micro variant form (OsHV-1 µvar) are an important pathogen of *C. gigas* (Davison et al., 2005; Segarra et al., 2010). Over the last two decades, this virus has caused severe mortality outbreaks (Friedman et al., 2005; Burge et al., 2006; Segarra et al., 2010; Jenkins et al., 2013; Mortensen et al., 2016), resulting in over 90% mortality and the

death of millions of oysters worldwide (Friedman et al., 2005; Burge et al., 2006; King et al., 2019c).

Several techniques, such as changing husbandry procedures (e.g., increasing oyster cultivation height), immersing spat at particular time frames to avoid OsHV1 infection, and breeding disease resistant oysters, have been used to address the effect of OsHV-1 microvar, with varied degrees of success (Degremont, 2011; Paul-Pont et al., 2013; Whittington et al., 2015; Carrasco et al., 2017). Oyster genetic lines with higher survival rates after exposure to OsHV-1 are often used in breeding programs (Degremont, 2011, 2013; Degremont et al., 2015b; Camara et al., 2017). While these breeding projects have had some success, resistant families nevertheless suffer from varied levels of mortality (juvenile oysters 5–19%; larvae up to 86%) (Degremont, 2011; Degremont et al., 2013, 2016b), and the mechanism(s) underlying resistance are frequently difficult to detect.

Several authors have described the physiological and immunological processes underlying OsHV-1 and OsHV-1 µvar resistance in selectively bred oysters (Sauvage et al., 2009; Degremont, 2011, 2013; Azéma et al., 2015; Degremont et al., 2015a, 2016a, b). Higher oyster size and weight are linked to increased infection resistance, although the reason for this is still unknown (Degremont, 2013; Degremont et al., 2015b). According to other investigations resistant oysters have a higher capacity to eliminate OsHV-1 from their tissues and suppress viral multiplication (Degremont, 2011; Segarra et al., 2014). When compared to susceptible oysters, resistant oysters appear to have a stronger potential to trigger autophagy genes when infected by OsHV-1 from an immunological standpoint (Moreau et al., 2015).

Following OsHV-1 µvar infection, the overall bacterial load, including the *Vibrio* community, is greatly raised, and this elevation is required to cause death (De Lorgeril et al., 2018). OsHV-1 infection promotes immune related suppression in the oyster host, allowing opportunistic

bacteria (including *Vibrio* species) to infect the oyster (Segarra et al., 2010; Garcia et al., 2011; Jenkins et al., 2013; Keeling et al., 2014; De Lorgeril et al., 2018). Studies in other species of oysters have linked the host microbiome to immune system modulation, implying that it is important for host defense and general health as well as affecting host behavior (King et al., 2019c). The oyster microbiome is another significant component that needs greater attention, to provide understanding of the differential microbial community between healthy and disease animals.

1.4 *Arcobacter*-like Species in Shellfish

Arcobacter-like bacteria are gram-negative, rod-shaped curved bacteria commonly found in food and water (Collado and Figueras, 2011; Salas-Massó et al., 2016; Venâncio et al., 2022). The genus *Arcobacter* was created by Vandamme et al. (1991) with species included in the genus *Campylobacter,* and since then the genus has expanded with the publication of several additional novel species (Collado and Figueras, 2011), encompassing a total of 34 species in which 30 are validly published (Alonso et al., 2020; Venâncio et al., 2022). The majority of these species have been recovered from shellfish (Salas-Massó et al., 2016; Rahman et al., 2020). Based on comparative genomic analysis, in 2017, the Family *Arcobacteraceae*, within the class *Campylobacteria*, order *Campylobacterales* was created to accommodate the genus *Arcobacter* Waite et al. (2017). The relationship among the species of this genus was then reconstructed using genomic and phylogenetic analysis and the known species were divided into six named genera i.e., *Aliarcobacter*, *Pseudarcobacter*, *Malaciobacter*, *Halarcobacter* and *Poseidonibacter* (and a candidate genus, ‘Aquimarinus’, missing for formal description), remain classified only as an *Arcobacter* species *Arcobacter nitrofigilis* (Perez-Cataluña et al., 2018). But this reclassification was rebutted based on the reasons that species in genus *Arcobacter* are phenotypically, genomically, and phylogenetically similar (On et al., 2020).

Hence, the taxonomic classification of *Arcobacter*-like species is controversial and still under discussion.

Arcobacter-like species are widely distributed in nature and found in water such as ground water, surface water, seawater, sewage and wastewater, food, vegetables, food processing plants, dairy products, meat, shellfish, human and animals (Snelling et al., 2006; Collado and Figueras 2011; Hsu and Lee 2015; Salas-Massó et al., 2016; Ramees et al., 2017; Rahman et al., 2020; Mateus et al., 2021; Venâncio et al., 2022). Several *Arcobacter*-like species are considered enteropathogenic and zoonotic pathogens and has been recovered from human and animal suffering from disease like diarrhea, abortion, and enteritis (Ho et al., 2006; Collado and Figueras 2011; Van den Abeele et al., 2014; Remees et al., 2017; Perez-Cataluña et al., 2018). *Aliarcobacter butzleri* has been classified as a serious risk to humans in 2002 by the International Commission on Microbiological Specifications for Foods (ICMSF, 2002). Several other species like *A. skirrowii*, *A. cryaerophilus*, *A. lanthieri*, *A. thereius* and *Malaciobacter mytili* have also been found associated with human illnesses such as vomiting, nausea, abdominal pain, fever, and bacteremia (Venâncio et al., 2022).

Water, shellfish, and animal-derived foods have been linked as possible reservoirs and transmission channels for potentially pathogenic *Arcobacter*-like species (Collado and Figueras 2011; Hsu and Lee 2015; Mottola et al., 2016; Perez-Cataluña et al., 2018). *Arcobacter*-like spp. are highly prevalent in poultry, followed by seafood, pork, dairy foods, lamb, beef, and rabbit, respectively (Salas-Massó et al., 2019).

1.5 Role of *Arcobacter*-like Species in Animal Production

Due to the increase rate of incidence, food-borne illnesses are now a days considered significantly important world widely (Ramees et al., 2017). *Arcobacter*-like spp. are considered important food-borne bacterial pathogen like other bacteria such as *Bacillus cereus*,

Salmonella, *Staphylococcus*, *Clostridium perfringens*, *Clostridium botulinum*, *Escherichia coli*, *Yersinia enterocolitica*, and *Campylobacter* (Collado and Figueras, 2011; Calvo et al., 2013; Dhama et al., 2013a; Hsu and Lee 2015). The disease mechanisms of many of these pathogens have been explored while the virulence mechanisms of some of them, like for *Arcobacter*-like spp., has not yet been completely understood (Collado and Figueras, 2011; Ramees et al., 2017).

The first isolation of *Arcobacter* has been from the tissues of aborted bovine and porcine fetus (Ellis et al., 1977; 1978). In 2002, the ICMSF classified this bacterium as responsible of generating emerging zoonotic diseases of food origin (Collado and Figueras, 2011; Ramees et al., 2017). Later, *Arcobacter*-like spp. was also found responsible for several animal diseases like diarrhea, mastitis and abortion as well as found responsible for diarrhea, endocarditis, bacteremia, peritonitis, and gastroenteritis in humans (Jiang et al., 2010; Collado and Figueras 2011; Figueras et al., 2014; Ferreira et al., 2016; Lameei et al., 2022; Kobayashi et al., 2022). They were also isolated from the blood samples from individuals who had gangrenous appendicitis and cirrhosis (Lau et al., 2002).

As commented the three *Arcobacter*-like species most frequently associated with clinical cases are *Aliiarcobacter butzleri*, *A. cryaerophilus*, and *A. skirrowii* (Collado and Figueras 2011; Ramees et al., 2017). There is debate over the pathophysiology and pathogenicity of *Arcobacter*-like species because they are regularly isolated from the feces of healthy animals and people and do not satisfy Koch's postulate (Shah et al., 2011; Ongor et al., 2004; Houf and Stephan 2007; Figueras et al., 2014).

Arcobacter has been found in healthy cattle, sheep, pigs, and wild boars (Ho et al., 2006a, 2006b; De Smet et al., 2011; Patyal et al., 2011; Ferreira et al., 2016; Kerkhof et al., 2022). According to numerous studies poultries serve as a reservoir and source of transmission for the

Arcobacter-like spp. infection along with other sources (Collado and Figueras 2011; Ramees et al., 2017). *Arcobacter*-like spp. has been linked to poultry intestines, which raises the possibility of further contamination in slaughterhouses during carcass processing (Ho et al., 2008a). Slaughterhouses are considered another source of the transmission of Arcobacter-like infections. It has been found that despite the disinfection procedures properly followed *Arcobacter*-like spp. still persisted in the system (Collado et al., 2010; Collado and Figueras 2011; Elmali and Can 2017; Khodamoradi and Abiri 2020). Additionally, *Arcobacter*-like spp. have been discovered in plants, water, and a variety of animal feeds, including milk, pigs, cattle, and seafoods (Collado and Figueras 2011; Ramees et al., 2017; Di Noto et al., 2018; Parisi et al., 2019; Salas-Massó et al., 2019). According to several studies (Snelling et al., 2006; Collado et al., 2010; Collado and Figueras 2011; De Smet et al., 2010; González and Ferrus, 2011; Rahimi 2014), infected meat and water help the disease spread. It has been determined that *Arcobacter*-like isolates collected from different parts of the world have a higher level of genetic diversity and an increase in antibiotic resistance (Ferreira et al., 2016; 2019).

1.6 Isolation of *Arcobacter*-like Species from Shellfish

Arcobacter-like spp. isolation is regarded as the best method for diagnosing and characterization of novel *Arcobacter*-like spp. and studying its role in a specific environment (Atabay and Corry, 1998; Aydin et al., 2007; Rahimi, 2014; Ramees et al., 2014b). Different growth media have been used for the cultural isolation of *Arcobacter*-like species (De Boer et al., 1996; Atabay and Corry, 1998; Johnson and Murano, 1999b; Ohlendorf and Murano, 2002; Scullion et al., 2004; Collado and Figueras, 2011; Ahmed and Balamurugan, 2013; Rahimi, 2014; Ramees et al., 2014b; Salas-Massó et al., 2016; Rahman et al., 2020). The culture method established by Houf et al. (2001) is the most widely used and includes a mixture of antibiotics, CAT (Cefoperazone, Amphotericin B, and Teicoplanin) in the broth enrichment media to suppress the growth of unwanted bacteria in a microaerophilic condition.

Despite the variety of isolation methods available, there is no accepted standard method for isolating *Arcobacter*-like spp. (Shah et al., 2011; Silha et al., 2021). This limiting feature, together with the complexity of the method, may cause many significant cases to escape unnoticed, underestimating the incidence and epidemiological status of Arcobacters, which also makes it more difficult to evaluate the results as published in prior investigations (Salas-Massó et al., 2016; Ramees et al., 2017; Rahman et al., 2020).

1.7 *Arcobacter*-like Species Diagnosis

Like *Campylobacter* species, *Arcobacter*-like species are fastidious and biochemically inert, which makes phenotypic differentiation challenging (Hilton 2004; Bhandari 2006; Ammar and Al-Habaty, 2015; Goni et al., 2017). As a result, polymerase chain reaction (PCR)-based identification techniques, particularly multiplex PCR for the genus and the species have been developed (Houf et al. 2000; Douidah et al. 2010; Ramees et al. 2014a) and are being used for their specific detection and identification (Brightwel et al., 2007; Douidah et al., 2010). The Arcobacter 23S qPCR (Hausdorf et al., 2013) is another rapid test to detect *Arcobacter*-like spp. in the samples. Besides that, the Arcobacter 23S v-qPCR (Viable qPCR) is another method in which a DNA intercalating dye e.g., propidium monoazide (PMA) is used to detect and count the viable cells of *Arcobacter*-like spp. in the samples (Salas-Massó et al., 2019). To improve the sensitivity and specificity of the currently employed diagnostic techniques, more study is required in the field of Arcobacters diagnostics.

2. INTEREST AND OBJECTIVES

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The epidemiology and taxonomy of the *Arcobacter*-like species was reviewed previously by Luis Collado (2010) and Alba Perez-Catalunya (2018) in their Ph.D. thesis from this group. Arturo Levican Asenjo in his Ph.D. studied the sanitary importance of *Arcobacter*-like species (Levican, 2013) and build the basis of an initial epidemiology study of *Arcobacter*-like spp. in shellfish exposed to fecal polluted marine and brackish water, concluded by Nuria Salas-Massó (2019) in her Ph.D. thesis. In the latter, it was discovered a great diversity of *Arcobacter*-like spp. in the shellfish of the Ebro Delta (Spain), using for the first time the addition of salt (NaCl) to the enrichment culture media. However, the role of these *Arcobacter*-like spp. in the cultivated shellfish and its mortality was not studied. Considering, that *Arcobacter*-like spp. have been found by other authors in association with the microbiome of shellfish and in mortality episodes (Lokmer and Mathias Wegner, 2015; Lasa at al., 2019) there is a clear interest, to clarify the role of these microbes in the shellfish health and, therefore, this is one of the objectives of the present Ph.D. thesis.

Considering the great efficiency of adding a common ingredient like NaCl to the media broth for the isolation of *Arcobacter*-like spp. from shellfish and marine compartments, it was considered important to evaluate the effect of different not yet tested ingredients for the growth and recovery of *Arcobacter*-like spp. from the marine environment. To that purpose, a new enrichment broth media was developed by adding different constituents to the Arcobacter enrichment broth, such as artificial seawater, to mimic the conditions of the natural habitats of *Arcobacter*-like species.

The Pacific oyster, *Crassostrea gigas* is one of the main shellfish species produced in the Ebro Delta, on the Mediterranean coast of Catalonia (Spain). However, the production of the *C. gigas* has been affected by extreme changes of environmental conditions, such high summer

temperatures, and the outbreak of different diseases, mainly of viral and bacterial origin, and of unknown etiology. The Ostreid herpes virus 1_ microvariant (OsHV-1 µvar) is the main viral pathogen which causes disease and mortality in juvenile *C. gigas* in different regions of the world, including the study area at the Ebro Delta. Nevertheless, *C. gigas* is also affected by other bacterial pathogens, consisting of several *Vibrio* species such as *Vibrio aestuarianus* and *V. splendidus* clade spp. Apart from the mentioned viral and bacterial pathogens, *Arcobacter*-like spp. has also been identified from both shellfish and seawater in the *C. gigas* of the study area, as reported by Levican (2013) and Salas-Massó (2019) in their thesis and publications (Levican et al., 2012; 2015; Salas-Massó et al., 2016; 2018; 2019). Subsequently, the epidemiological study of OsHV-1, *V. aestuarianus*, *V. splendidus* clade and *Arcobacter*-like spp. in the unexplained mortality episodes of *C. gigas* in the Ebro Delta, is the second objective of the present Ph.D.

Several studies have reported the rise of *Arcobacter*-like spp. in the hemolymph and tissue of the *C. gigas* from the mortality episodes using molecular methods such as the microbiome and next generation sequencing. It was reported that these bacteria may have a potential role in causing the mortality in *C. gigas*. However, the potential roles of these bacterial spp. in the mortality of *C. gigas*, was not studied earlier using *in vivo* challenge experiments. So, the third objective was to determine the potential role of *Arcobacter*-like spp. in the mortality of *C. gigas*.

Genomic studies of *Arcobacter*-like spp. revealed that they can carry many putative virulence genes and immune suppressing genes (Martinez-Malaxetxebarria et al., 2022; Barel and Yildirim, 2023). The virulence genes were suspected to have a role in weakening the immune system of the host where these bacteria carrying the virulence genes, along with other colonizers, could cause mortalities in *C. gigas*. So, the fourth objective included in this Ph.D. was developed to investigate the interaction of *Arcobacter*-like spp., isolated in this work

during mortality episodes, with the hemocytes of two important shellfish species i.e., *Crassostrea gigas* and *Mytilus galloprovincialis* by *in vitro* challenge experiments.

The overall aim of the present thesis is to study and provide new insights of the effect of the *Arcobacter*-like species on shellfish health and its potential role in causing harm to shellfish. To accomplish this aim, we developed the following specific objectives:

1. To improve the enrichment broth media for the growth of *Arcobacter*-like species. **3.1 Study 1**
2. To find out the epidemiology of *Arcobacter*-like spp., *Vibrio aestuarianus, Vibrio splendidus* clade and OsHV-1 (Oyster Herpesvirus type 1) in cultured Pacific oysters, (*C. gigas*) from Ebro Delta, Spain. **3.2 Study 2**
3. To develop specific detection methods (PCR and qPCR) to improve screening of selected *Arcobacter*-like strains. **3.2 Study 2**
4. To determine the potential role of selected strains of *Arcobacter*-like spp. in causing harm in Pacific oysters, *C. gigas* by means of *in vivo* studies. **3.3 Study 3**
5. To study *in vitro* the interactions between selected strains of *Arcobacter*-like spp. with important shellfish cultures species. **3.4 Study 4**

3. RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

3.1 Study 1: Improved culture enrichment broth for isolation of *Arcobacter*-like species from the marine environment.

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RESULTS AND DISCUSSION

Summary

Arcobacter-like species are gram-negative, slightly curved, rod-shaped bacteria found in many different environments, such as water, sewage, and food products like vegetables, dairy products, and marine animals. The paper deals with the need for new cultivation strategies to enhance the isolation and efficient growth of *Arcobacter*-like species associated with shellfish or their environment. The work aimed to develop a culture enrichment broth to improve the isolation of *Arcobacter*-like species from the marine environment. The study tested the growth of 14 reference strains on 24 different formulations of enrichment broths, based on the organic content (fresh or lyophilized oyster homogenates either alone or in combination with standard media), suspended in artificial seawater (ASW) or 2.5% NaCl. The growth was monitored by means of optical density (OD 420_{nm}) measurements and compared with that of a standard Arcobacter broth, running in parallel.

The results indicated that shellfish-associated *Arcobacter*-like species growth was significantly higher in Arcobacter broth + 50% ASW and in the same media supplemented with lyophilized oysters. Consequently, the study successfully developed an improved enrichment broth for enhancing the isolation and growth of *Arcobacter*-like species from marine and shellfish matrices, highlighting the importance of mimicking the original environmental conditions. The results suggest that marine *Arcobacter*-like species have specific nutritional requirements. This approach should render more efficient isolation and cultivation of marine-derived *Arcobacter*-like species in the future and could help to enhance the description of diversity of species obtained from shellfish. Further work with lyophilized oyster material supplementing the media could support pathogenicity and biochemical studies.

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Improved culture enrichment broth for isolation of *Arcobacter*-like species from the marine environment

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***Arcobacter*-like species are found associated with many matrices, including shellfish in marine environments. The culture media and conditions play a major role in the recovery of new *Arcobacter*-like species. This study was aimed to develop a culture media for isolation and enhanced growth of *Arcobacter*-like spp. from marine and shellfish matrices. For this purpose, 14 different *Arcobacter*-like spp. mostly isolated from shellfish, were grown in 24 different formulations of enrichment broths. The enrichment broths consisted of five main groups based on the organic contents (fresh oyster homogenate, lyophilized oyster either alone or in combination with other standard media), combined with artificial seawater (ASW) or 2.5% NaCl. Optical density (OD_{420nm}) measurements after every 24 h were compared with the growth in control media (Arcobacter broth) in parallel. The mean and standard deviation were calculated for each species in each broth and statistical differences ($p < 0.05$) among broths were calculated by ANOVA. The results indicated that shellfish-associated *Arcobacter*-like species growth was significantly higher in Arcobacter broth + 50% ASW and the same media supplemented with lyophilized oysters. This is the first study to have used fresh or lyophilized oyster flesh in the enrichment broth for isolation of shellfish-associated *Arcobacter*-like spp.**

Arcobacter-like species are gram negative, slightly curved, rod-shaped bacteria. The genus *Arcobacter* was separated from the genus Campylobacter in 1991 by Vandamme et al.[1] and rapidly enlarged with the description of many new species, the majority of them described from shellfish, embracing in 2017 a total of 27 species. The recent taxonomic re-classification grouped these species in seven different genera, leaving only the type species *Arcobacter nitrofigilis* in the original genus[2]. For the purpose of this study, we will mainly either use the original species names or refer to all the species as *Arcobacter*-like species. These species are fastidious organisms and were differentiated from the campylobacters due to their ability for growth in the presence of oxygen and at significantly lower temperatures i.e. 15–30 °C[3,4].

Arcobacter-like species are cosmopolitan in their nature because they are found in different environments, such as water, septic tanks[5,6], human faeces[7], sewage, water treatment plants[6,8–13], food products[9,14], vegetables and vegetables processing plants[4,15,16]. They are also found in dairy products and buffalo milk[17,18]. They are also isolated from meat in pork and beef[8,19] slaughterhouses in Portugal[20]. They are commonly associated with seafood[3,21,22] such as shellfish[23–26], abalone[27], lobster[28], mussels, oysters, clams and seawater[29,30]. They are also reported from zooplankton, such as copepods[31], as symbionts with other animals, for example as endo-cytobiont of amoeba[32] and in mutualism with marine animals[33]. They had been sporadically found in association with reptiles[34] and dogs mouth`s and faeces[35].

Despite their diversity and isolation from different environments, *Arcobacter*-like species are also considered zoonotic and enteropathogenic, since they have been isolated during several infections and disease episodes both in humans and in animals[36]. Many *Arcobacter*-like species, now included in the new genus *Aliiarcobacter* i.e., *A. butzleri, A. cryaerophilus, A. skirrowii, A. cibarius* and *A. thereius* were considered important pathogens linked to gastrointestinal disease, causing diarrhoea in humans and abortion and enteritis in animals[2,3,21,37–39]. While, *A.*

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butzleri has been classified as an emerging food pathogen and serious health hazard to humans by International Commission on Microbiological Specifications for Foods (ICMSF)[40] in 2002. A recent study reported for the first time that the species of marine origin *Arcobacter mytili* (now named *Malaciobacter mytili*) as responsible for a case of bacteraemia[41], suggesting there exists a virulence potential by these bacteria in humans.

Therefore, water, food products of animal origin and shellfish have been considered as reservoirs and potential transmission routes for potentially pathogenic *Arcobacter*-like species[3,26,42]. The prevalence rate of *Arcobacter*-like spp. from food products, from highest to lowest, is from poultry, seafood, pork meat, dairy products, lamb, and beef followed by rabbit[43,44].

Despite some authors having underlined the need for developing standardized culture media, until now, no official standard protocol exists for the isolation of *Arcobacter*-like spp. from any specific kind of products or environment[3]. Moreover, a pre-enrichment step is needed to enhance the isolation of *Arcobacter*-like species and therefore isolation is time-consuming, requiring at least 72–96 h for the growth of pure bacterial cultures[30,45]. During isolation of bacteria on synthetic media, some bacteria may undergo a Viable But Non-Culturable (VBNC) state failing to grow, and are therefore not detected due to insufficient nutrients, growth promoting factors, or to other unknown reasons[46].

Out of 27 *Arcobacter*-like species, 18 have been isolated from marine environments, and 9 of those from shellfish. Regardless, we still lack complete knowledge of the diversity and presence of commensal, as well as pathogenic species found in marine invertebrates[27,30]. The increasing number of *Arcobacter*-like species isolated from shellfish by using different isolation approaches, such as enrichment or addition of salts[27,30], clearly indicates that there is still great potential for isolation of new *Arcobacter*-like species in nature, and specifically from marine environments. A recent microbiome study of the pacific oyster (*Crassostrea gigas*) demonstrated the presence of *Arcobacter*-like species in relatively higher number along with known oyster pathogens, such as *OsHV-1* and *Vibrio aestuarianus*[47]. This highlights the importance of studying in detail the interaction between the *Arcobacter*-like species and shellfish species and their possible role in the pathogenicity in *C. gigas*[48].

To be able to isolate *Arcobacter*-like species from different sources efficiently it is crucial to enrich bacteriological culture media by adding specific nutrients in such a way that mimics the original environmental conditions. To date, several enrichment broths and selective media have been used for the isolation and selective culturing from foods. The broth media used are: Arcobacter selective broth (ASB) I[49], Ellinghausen-McCullough-Johnson-Harris Polysorbate 80 broth (EMJH P80)[50], Arcobacter enrichment medium (AM)[51], Johnson and Murano broth (JMB)[52], ASB II[53] and Arcobacter broth + 2.5% NaCl[30]. Among the latter media the Arcobacter broth + 2.5% NaCl produced 40% more positive samples and provided a higher diversity of known and new species, 11 and 7 respectively[30]. This remarkable discovery of new species was made possible just by adding NaCl to the standard Arcobacter broth and by sub-culturing on marine agar.

The isolation of several new *Arcobacter*-like species from shellfish increased when new cultivation strategies mimicked the original environmental conditions more closely[29,30,54]. In this work, we are testing the enhanced cultivation of *Arcobacter*-like species associated with shellfish or their environment, by formulating culture media containing shellfish matrices and different percentages of artificial seawater (ASW), with the aim of developing one or more improved enrichment broths to enhance their efficient growth for functional and taxonomic work.

Results

Physical appearance of enrichment broth media supplemented with oyster homogenate or lyophilized oyster. The enrichment broth supplemented with oyster homogenate (OH) was of dark green colour and comparatively turbid and more opaque after autoclaving the broth. While enrichment broth media supplemented with lyophilised oyster was transparent like other conventional broths and comparatively pale in colour compared to the control enrichment broth i.e. Arcobacter broth, after autoclaving.

Evaluation of *Arcobacter*-like species growth in different enrichment broth group. *Growth of **Arcobacter**-like species in conventional enrichment broth with/or without artificial seawater.* All 14 of the strains tested showed growth in all 6-enrichment broths of this group (Fig. 1) although with different ODs. The OD_{420nm} range between 0.4 and 0.8 showed a linear correlation (data not shown), which indicated that the growth of the species reached the exponential phase. A significantly ($p < 0.05$) higher growth in ABSW was observed for *H. bivalviorum* F159-36 in relation to any other *Arcobacter*-like specie used in this study. The strains of the Candidatus '*A. salitolerans*' F166-33, and the strains of the species *M. marinus* W132-33, *A. butzleri* F170G17, and *A. hispanicus* F164-18 also showed a comparatively higher growth in the ABSW enrichment broth than in the control AB broth and other enrichment broth in this group, but these differences were not statistically significant ($p > 0.05$). A higher growth, but without significant difference, was also observed for *M. halophilus* F166-43, *A. mediterraneus* W143-33, and *M. canalis* F167F33 in ABSW100 as compared to AB (Control broth). *A. butzleri* LMG 10828^{T} showed significantly higher growth in MB (Marine broth). The strains of *P. aquimarinus* F185-17 and *M. canalis* F138-33^{T} showed significantly higher growth in ABNC compared to control broth (AB). The growth *A. nitrofigilis* W111-35 and *P. aquimarinus* W63^{T} showed uniform growth but lower than 0.4 at OD_{420nm} (No significant difference). The species *A. butzleri* LMG 10828^{T}, *A. hispanicus* F164-18 and *P. aquimarinus* F185-17 showed lower growth at the higher salt content in ASW compared to AB control.

Growth of *Arcobacter*-like spp. in oyster homogenate with conventional broth media group. This group consisted of five different types of broth. The results (Fig. 2) from this media group demonstrated lower growth than from group 1, with an OD_{420nm} below 0.8. The growth of *Arcobacter*-like spp. was comparatively higher in the enrichment broth ABOHSW than in ABOHN, but the difference among the growth was not significant ($p > 0.05$) when compared with the AB control broth media. Candidatus '*A. salitolerans*' F166-33, *M. marinus* W132-33,

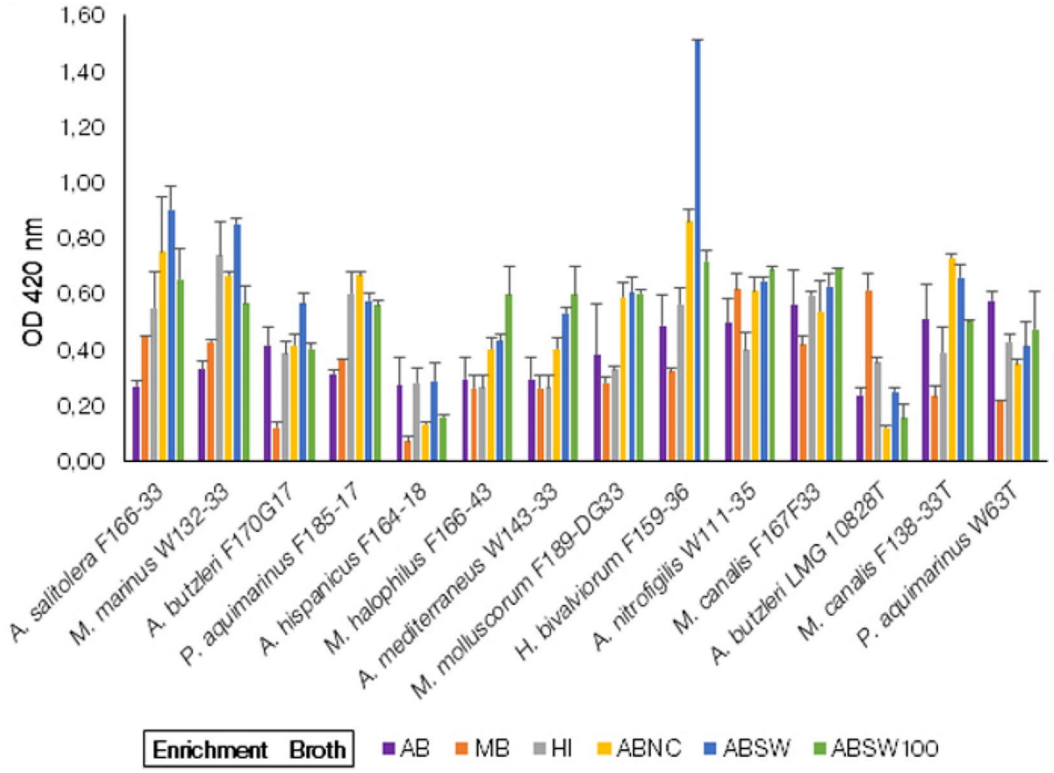


Figure 1. Growth of *Arcobacter*-like spp. in the conventional broth media. AB = Arcobacter Broth (Control Enrichment Broth); MB = Marine Broth; HI = Heart Infusion; ABNC = Arcobacter Broth + 2.5% NaCl; ABSW = Arcobacter Broth + 50% ASW; ABSW100 = Arcobacter Broth + 100% ASW. (Note: The strains with a T in the last are Type strains).

P. aquimarinus F185-17, *A. hispanicus* F164-18, *A. mediterraneus* W143-33, *M. molluscorum* F189-DG33, *H. bivalviorum* F159-36, *A. nitrofigilis* W111-35, *M. canalis* F167F33, and *A. butzleri* LMG 10828 ^{T} showed comparatively higher, though not significant growth, in the control AB media. The growth of all *Arcobacter*-like species was lower in ABOHSW100 enrichment broth except for *A. butzleri* F170G17, *M. halophilus* F166-43, *A. mediterraneus* W143-33 and *A. butzleri* LMG 10828 ^{T} which favour higher salinity and have comparatively higher growth in 100% ASW. The growth of most *Arcobacter*-like spp. i.e. Candidatus '*A. salitolerans*' F166-33, *M. molluscorum* F189-DG33, *H. bivalviorum* F159-36, *A. nitrofigilis* W111-35, *M. canalis* F167F33, *A. butzleri* LMG 10828 ^{T}, *M. canalis* F138-33 ^{T} and *P. aquimarinus* W63^{T} was higher (no significant difference as $p > 0.05$) in ABOHSW than ABOHN. While *M. marinus* W132-33, *A. butzleri* F170G17, and *P. aquimarinus* F185-17 have comparatively higher growth (no significant difference as $p > 0.05$) in ABOHN (Arcobacter broth + 2.5% NaCl supplemented with Oyster homogenate) than ABOHSW. There was no significant difference among all 14 *Arcobacter*-like species when compared with control AB enrichment broth.

Growth of *Arcobacter*-like species in oyster homogenate enrichment broth group. The enrichment broth group consisted of four enrichment broth media prepared only with oyster homogenate i.e. OH, OHNC, OHSW and OHSW100, without addition of any conventional media components or carbon source. Almost all the strains showed growth (Fig. 3) in these different enrichment broth media but with lower levels than the control broth AB. One exception was for the strain *A. butzleri* LMG 10828 ^{T}, which showed comparatively higher growth than in the control AB broth in comparison to all other strains, but the growth was not significantly higher ($p > 0.05$). Many *Arcobacter*-like species i.e. *M. marinus* W132-33, *P. aquimarinus* F185-17, *M. molluscorum* F189-DG33, *H. bivalviorum* F159-36, *A. nitrofigilis* W111-35, and *M. canalis* F167F33 showed a higher growth in the control broth AB. The strains of *A. butzleri* F170G17, *A. hispanicus* F164-18, *M. halophilus* F166-43, *A. mediterraneus* W143-33, *A. butzleri* LMG 10828 ^{T}, *M. canalis* F138-33 ^{T} and *P. aquimarinus* W63^{T} could grow in OH with or without salts even in the absence of any additional carbon source from the media components.

Growth of *Arcobacter*-like species in conventional broth media supplemented with powder from lyophilized oysters. This media group consisted of five types of enrichment broth media prepared by adding to the conventional broth media powder from lyophilized oysters. In this group of enrichment broths ABLSW was the most favourable enrichment broth followed by ABLNC in which almost all *Arcobacter*-like species showed significantly higher growth ($p < 0.05$) compared to the control AB media except for *A. butzleri* F170G17, *A. butzleri* LMG10828^{T}, *A. hispanicus* F164-18 and *P. aquimarinus* W63^{T} (Fig. 4). A higher growth ($p < 0.05$) was obtained in ABLSW by Candidatus '*A. salitolerans*' F166-33, *M. marinus* W132-33, *P. aquimarinus* F185-17, *M. halophi-*

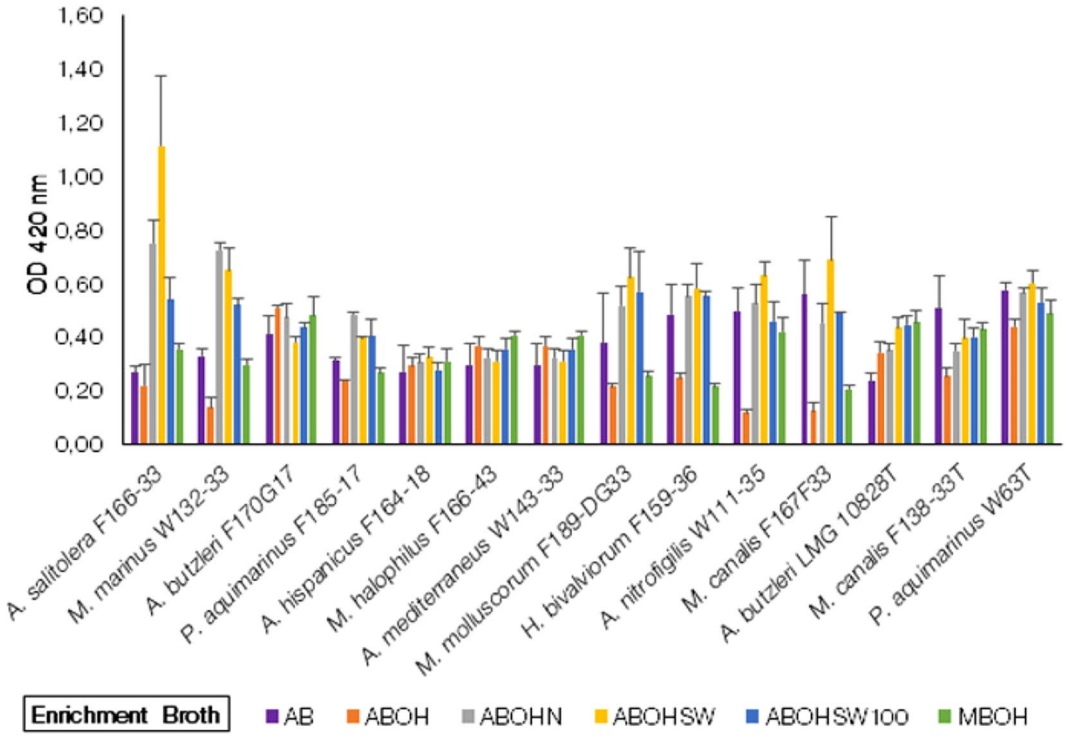


Figure 2. Growth of *Arcobacter*-like spp. in the conventional broth media supplemented with oyster homogenate. AB = Arcobacter Broth (Control Enrichment Broth); ABOH = Arcobacter Broth + Oyster Homogenate; ABOHN = Arcobacter Broth + Oyster Homogenate + 2.5% NaCl; ABOHSW = Arcobacter Broth + Oyster Homogenate + 50% ASW; ABSW100 = Arcobacter Broth + Oyster Homogenate + 100% ASW; MBOH = Marine Broth + Oyster Homogenate. (Note: The strains with a "T" suffix are Type strains).

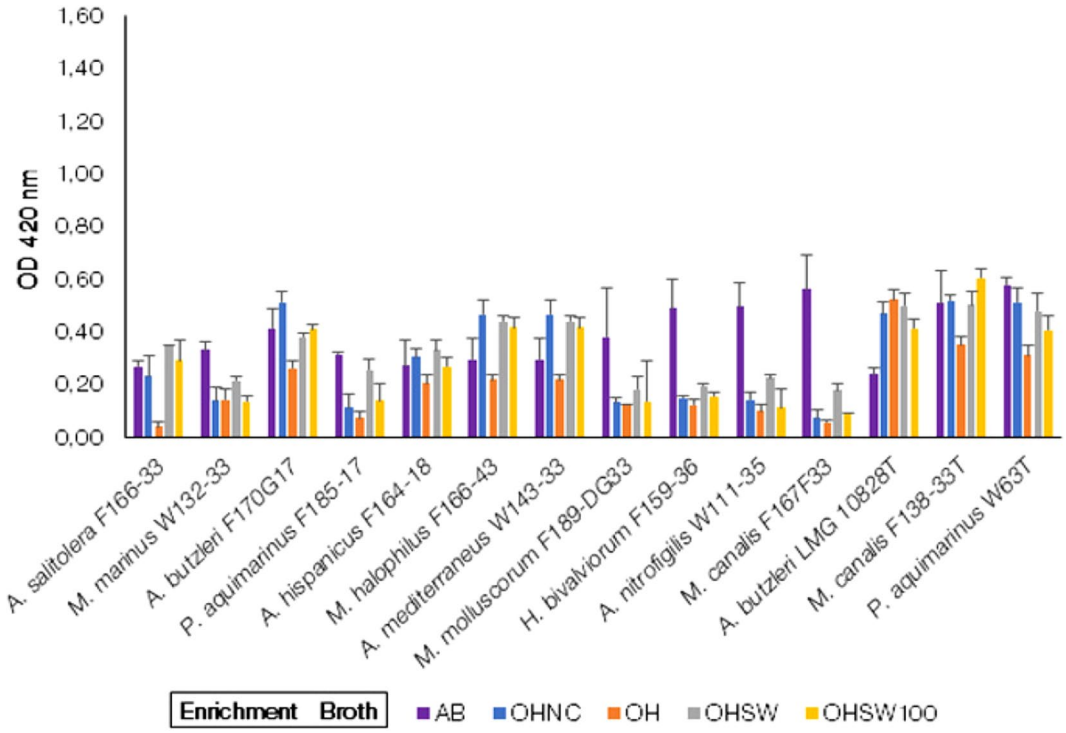


Figure 3. Growth of *Arcobacter*-like spp. in the enrichment broth media prepared from oyster homogenate. AB = Arcobacter Broth (Control Enrichment Broth); OH = Oyster Homogenate + Distilled H_2O; OHNC = Oyster Homogenate + 2.5% NaCl; OHSW = Oyster Homogenate + 50% ASW; OHSW100 = Oyster Homogenate + 100% ASW. (Note: The strains with a "T" suffix are Type strains).

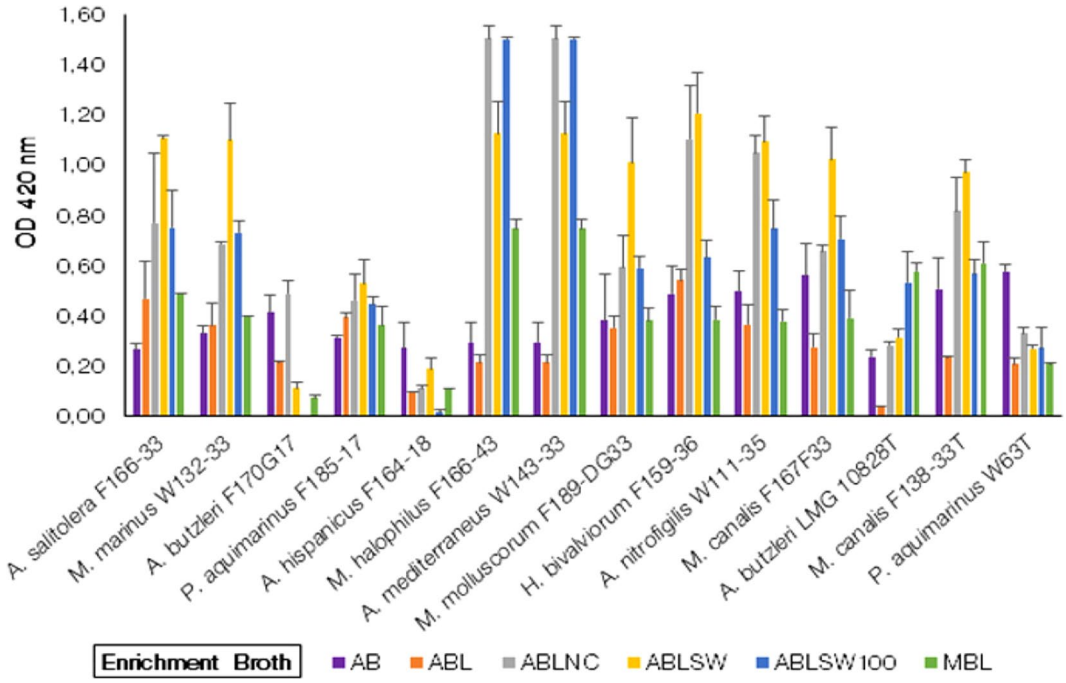


Figure 4. Growth of *Arcobacter*-like spp. in conventional broth media supplemented with lyophilized oyster. AB = Arcobacter Broth (Control Enrichment Broth); ABL = Arcobacter Broth + Lyophilized Oyster; ABLNC = Arcobacter Broth + Lyophilized Oyster + 2.5% NaCl; ABLSW = Arcobacter Broth + Lyophilized Oyster + 50% ASW; ABLSW100 = Arcobacter Broth + Lyophilized Oyster + 100% ASW; MBL = Marine Broth + Lyophilized Oyster. (Note: The strains with a "T" suffix are Type strains).

lus F166-43, *A. mediterraneus* W143-33, *H. bivalviorum* F159-36, *A. nitrofigilis* W111-35, *M. canalis* F167F33 and *M. canalis* F138-33 ^{T} compared to the growth in the control AB broth. ABLNC was the 2nd most favourable broth media in which Candidatus *'A. salitolerans'* F166-33, *M. marinus* W132-33, *M. halophilus* F166-43, *A. mediterraneus* W143-33, *H. bivalviorum* F159-36, *A. nitrofigilis* W111-35 and *M. canalis* F138-33 ^{T} showed significantly ($p < 0.05$) higher growth. A significantly higher growth ($p < 0.05$) in ABLSW100 broth media was observed for *M. halophilus* F166-43 and *A. mediterraneus* W143-33 when compared to control AB broth.

Growth of *Arcobacter*-like species in salty water or artificial seawater supplemented with powder from lyophilized oysters. This group of media were prepared with lyophilized oyster powder in distilled water, alone or with a basal solution of 2.5% NaCl, 50% ASW or 100% ASW. This group consisted of four types of broth media. Growth was observed in almost all four types of broth included in this group for all *Arcobacter*-like species tested (Fig. 5) except *A. butzleri* F170G17, and *A. hispanicus* F164-18 which did not grow in any type of broth. The growth was similar or lower than in the control AB broth in all four types of enriched broth media for the strains of the species *M. marinus* W132-33, *P. aquimarinus* F185-17, *M. molluscorum* F189-DG33, *H. bivalviorum* F159-36, *A. nitrofigilis* W111-35, *M. canalis* F167F33, *A. butzleri* LMG 10828 ^{T}, *M. canalis* F138-33 ^{T} and *P. aquimarinus* W63^{T}. Comparatively higher growth, though not significantly different ($p > 0.05$), was observed in LOSW for the strain of Candidatus '*A. salitolera*' F166-33. The strains *M. halophilus* F166-43 and *A. mediterraneus* W143-33 only grew in LSW100 apart from control AB broth. There were not significant differences ($p > 0.05$) in growth among different species in this media group.

Discussion

Some *Arcobacter*-like species are considered emerging foodborne pathogens[3]. They were initially reported from terrestrial environments, food and dairy products but many were later on found associated with seafood[9,24,30]. Shellfish have been considered as reservoirs for pathogenic *Arcobacter*-like species for humans[26,44]. These gram-negative rod-shaped bacteria, previously known as the genus *Arcobacter*, have been recently re-classified into seven genera[2]. This new classification is partially supported by the different nutritional requirements for the growth of some of the new genera like the growth in the presence/absence of NaCl[30]. It had been demonstrated that the addition of salt to the culture media favoured the recovery of many new species from shellfish. However, to our knowledge so far it had not been evaluated if the addition of shellfish-based tissues and inter-valval liquid to the enrichment broth could be an organic component that would favour the growth, isolation and recovery of *Arcobacter*-like species from shellfish. For the isolation of bacteria from its natural reservoirs, it is always a strategy by environmental microbiologists to provide, with the best possibility, the same nutrients and environmental conditions found in the sampling site. Microbial interaction among microbial communities in nature and their metabolism is dependent on several parameters like pH, nutrients, osmotic pressure, temperature and

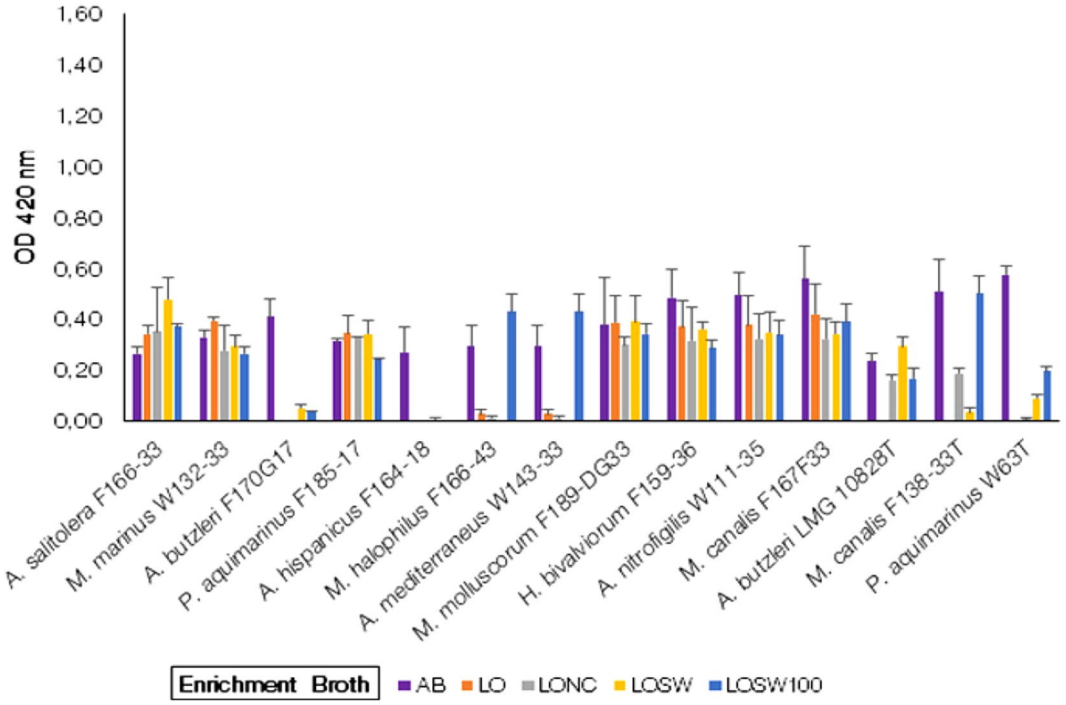


Figure 5. Growth of *Arcobacter*-like spp. in the broth media prepared with lyophilized oyster. AB = Arcobacter Broth (Control Enrichment Broth); LO = Lyophilized Oyster + Distilled H_2O; LONC = Lyophilized Oyster + 2.5% NaCl; LOSW = Lyophilized Oyster + 50% ASW; LOSW100 = Lyophilized Oyster + 100% ASW. (Note: The strains with a "T" suffix are Type strains).

several other factors[55]. The failure in isolation of bacteria in many culture media is attributed to inappropriate culture conditions[56]. Therefore, in order to improve isolation of *Arcobacter*-like spp. related to shellfish and/or their environment we aimed to incorporate into the standard Arcobacter broth media, a series of enrichments to better mimic the marine and the shellfish environment. The latter were assayed independently or combined and are the following: seawater (ASW), oyster homogenate (OH), lyophilized oyster (LO) and artificial seawater (ASW). Overall, there were 24 different combinations of enrichment broth, which were divided into five groups of enrichment broth media each with different basal ingredients. The composition of oyster meat is 77–83% moisture content, 9–13% proteins, 1–3% fats, and 1–5% glycogen as a main carbohydrate[57]. According to the work of Zhu et al.[58], Pacific oyster (*Crassostrea gigas*) contains 79–80% moisture and the composition by percent dry weight was 50–53% proteins, 3–5% fats, 16–22% glycogen and 9–10% ash after freeze drying.

The OH or LO materials were provided in a specific quantity in the enrichment broth in order to get an amount of approximately 0.5% organic content from oyster in combination with conventional enrichment broth (group b and d), or 1% organic contents from oyster (in group c and e enrichment broths). These percentages were calculated based on the organic contents of the conventional broth media i.e. Arcobacter broth, tryptic soy broth, heart infusion and marine broth. From the oyster, organic content 0.5% was used where there was an organic content added from the conventional broth media, while 1% was used in the absence of any organic source other than oyster. The different *Arcobacter*-like species grew in almost all types of broth media, but with large variation between the five groups of enrichment broth. The conventional broth group also comprised three derivatives of Arcobacter broth by adding salts, i.e., Arcobacter broth supplemented with 2.5% NaCl (ABNC), (used previously for isolation of marine *Arcobacter*-like spp[30,59].) and two concentrations of ASW i.e. 50% and 100% (ABSW and ABSW100 respectively). The growth *Arcobacter*-like spp. was comparatively higher in ABSW than previously used ABNC[30] except *P. aquimarinus* F185-17 and *M. canalis* F138-33 ^{T} where the growth was higher in ABNC.

The major non-essential amino acids were aspartic acid, glutamic acid, arginine, taurine and alanine, which makes about 50% of the total protein content in oyster meat. Leucine, valine, lysine, isoleucine, phenylalanine, methionine and threonine are also present as essential amino acids in the composition of oyster (*C. gigas*) meat. Saturated and unsaturated fatty acids like palmitic acid are also found in *C. gigas* meat. Calcium is the most abundant macro mineral followed in *C. gigas* by Mg along with Zn, Cu, Fe and Se[58]. The results obtained from these experiments shows that *Arcobacter*-like species nutritional requirements vary widely. The overview of results from all the species indicates that seawater has a very positive influence on enhancing growth of *Arcobacter*-like species in the conventional broth media group and in enrichment broth combined with LO (group 3a). The enhanced recovery of *Arcobacter*-like spp. by the addition of 2.5% NaCl in Arcobacter broth resulted in the recovery of 40% more *Arcobacter*-like spp.[30], compared to the previously used Arcobacter broth. Recently, a similar approach used Arcobacter broth + 75% ASW for the isolation of *Arcobacter*-like species from abalone (*Haliotis gigantea*) samples and this resulted in the recovery of two previously unknown *Arcobacter* isolates[27]. The higher growth shown by the species such as Candidatus '*A. salitolerans*' F166-33, *M. marinus* W132-33, *M. halophilus* F166-43,

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M. molluscorum F189-DG33, *H. bivalviorum* F159-36 and *M. canalis* F138-33 ^{T} in ABSW (group 1) compared to control broth AB, indicates that the growth of these species is significantly influenced by the presence or absence of seawater. These results clearly indicate that using seawater in the media preparation improve their isolation and enhance cultivation of *Arcobacter*-like species from marine environments. In addition to Na and Cl, other ions though collectively in extremely low concentrations, can be critically important for marine organisms[60]. Tyler et al.[61] demonstrated that 35 out of 96 marine bacterial isolates had been isolated from marine samples by adding all 4 major salts i.e. NaCl, $MgCl_2$, $MgSO_4$, and KCl in 1% (w/v) trypticase broth media. Neither of these isolates could be recovered in the absence of any of these salts. This may be due to the inability of marine bacteria to grow on the substrate or media combination provided as we have very little knowledge of the organic substrate and its concentrations available to those bacteria in the marine environment[62]. Marine bacteria need Na^+ for their growth, as the ion is necessary for enzyme activity, membrane stability, active transport, respiration-dependent primary Na^+-pumps and many other functions in bacterial cells[63–67]. The genome analyses of many human and animal bacterial pathogens show that they encode Na^+ based enzymes i.e. decarboxylases, oxidoreductases and permeases as well as membrane primary Na^+ pumps instead of, or in addition to H^+ pumps, and these might be important virulence factors for several pathogenic bacteria[68]. Further, the presence of ions can affect the mechanical properties of biofilms and serves as a cross linker in the attachment during biofilm formation[69,70]. The complete elemental requirements for cell growth (i.e. which elements and in which amounts/ concentration) for *Arcobacter*-like species remains an area for future studies.

Although, the 14 species used in this study (out of 29 *Arcobacter*-like species known) were originally isolated from the marine environment, we still lack complete knowledge on the diversity and presence of commensals or pathogenic species among those genera found in marine invertebrates[27]. The approaches followed by Salas-Masso et al.[30], Mizutani et al.[27] and Kim et al.[71] resulted in enhanced recovery of *Arcobacter*-like species from shellfish, abalone and food samples. These studies indicate that still there is a great potential for isolation of new species, especially from the marine environment.

The supplementation of shellfish tissue (OH and LO) into the enrichment broth significantly enhanced the growth of *Arcobacter*-like species (p value < 0.05) especially in the ABLSW enrichment broth (Arcobacter broth + Lyophilized Oysters + 50% ASW) when compared to the control broth AB. The supplementation of only LO did not improve the growth in the absence of 50% ASW, which may suggest a synergy between the ASW and the LO supplement for the enhancement of the recovery of strains from marine environments. While the lowest growth was observed in ABOH enrichment broth for most species, the addition of only OH into Arcobacter broth did not have a promising effect on the growth either. The control AB broth when supplemented with LO flesh (i.e. ABL broth) does not seem to improve growth, and even decreased growth for most of the species. However, when ABL was supplemented with salts such as 2.5% NaCl and 50% ASW, there were significant improvements in growth for most of *Arcobacter*-like spp. mentioned above.

Through successive studies, a more efficient broth ASB II (Arcobacter selective broth II) has been adopted for the enrichment of *Arcobacter*-like spp.[72]. However, fastidious microorganisms such as *Gemmata obscuriglobus* and *Gemmata massiliana* have been successfully grown using supplements, such as ground fresh sponge tissues, in culture media[56]. Similarly, the results herein indicate that Arcobacter broth + 50% ASW (ABSW) and Arcobacter broth + Lyophilized Oyster + 50% ASW (ABLSW) are a better enrichment broth for the isolation of *Arcobacter*-like species from shellfish and marine environments, as compared to the Arcobacter broth used in many studies. Therefore, these protocols should be adopted in future studies for the isolation of *Arcobacter*-like species from shellfish samples. Unidentified specific components of oyster flesh/ASW may contain key trace compounds necessary for fulfilling nutritional requirements and enhancing growth of some *Arcobacter*-like spp. The biochemical composition of *C. gigas* flesh is a rich source of proteins, essential and non-essential fatty acids, and macro and micro minerals[58]. Preferably, lyophilized oysters should be used in the media along with ASW, rather than oyster homogenate because this confers to the broth media a dark green colour and increases turbidity thereby obscuring observations.

The results obtained from this study suggest that marine-derived *Arcobacter*-like species have somewhat specific nutritional requirements. The approach used in this study should result in more efficient isolation and cultivation of marine-derived *Arcobacter*-like species in the future and could help enhance the description of diversity of species obtained from shellfish. These finding suggests that Arcobacter broth + 50% artificial seawater (ABSW) is the best enrichment broth medium for the growth of marine based *Arcobacter*-like spp. A more detailed work is required to refine the lyophilized oyster material used for supplementation and study the effect of different biochemical components in the lyophilized oyster on the growth of individual species or on the different genera as target groups.

Material and methods

Bacterial isolates. The experiment was performed with 14 different *Arcobacter*-like species that belonged to 5 out of 7 different genera according to the new classification of Perez-Catalunya et al.[2] shown in Table 1. Most bacterial isolates used in this study were previously isolated in IRTA (Sant Carles de la Rapita, Spain) and Universitat Rovira i Virgili (Reus, Spain) from shellfish or their water environment; type strains have been included for comparative purposes (Table 1). Out of 14 *Arcobacter*-like species used in this study, 13 species were isolated previously from Alfacs Bay and shellfish exposed to Poble Nou Channel Water and one *Arcobacter*-like Type strain was purchased from Spanish Culture Collection, CECT (Table 1). The strains were refreshed from − 80 °C glycerol stock suspensions on respective solid media i.e. marine agar and blood agar media and incubated aerobically for 48 h at 30 °C.

Strain name	Old name	Strain code in URV collection	Enrichment broth	Isolation media	Isolation source	Source of first report of the species
Candidatus *Arcobacter salitolerans*	Candidatus *Arcobacter salitolerans*	F166-33	Arcobacter CAT broth + 2.5% NaCl	Marine Agar	Oyster exposed to Poble Nou Channel Water	Oyster exposed to sewage water[43]
Aliiarcobacter hispanicus	*Arcobacter hispanicus*	F164-18	Arcobacter CAT broth	Blood Agar	Oyster (Alfacs Bay)	Oyster Alfacs Bay[2]
Halarcobacter mediterraneus	*Arcobacter mediterraneus*	W143-33	Arcobacter CAT broth + 2.5% NaCl	Marine Agar	Poble Nou Channel Water	Mussel Alfacs Bay[2]
Arcobacter nitrofigilis	*Arcobacter nitrofigilis*	W111-35	Arcobacter CAT broth + 2.5% NaCl	Marine Agar	Poble Nou Channel Water	Marshland plant roots[73]
Aliiarcobacter butzleri (Type)	*Arcobacter butzleri* (Type)	LMG 10828 ^{T}	Direct cultured on blood agar medium	Blood Agar	Man, with diarrhoea, faeces (USA, 1990)	Human and Animal diarrheal Samples [74]
Aliiarcobacter butzleri	*Arcobacter butzleri*	F170G17	Arcobacter CAT broth	Blood Agar	Oyster exposed to Poble Nou Channel Water	Human and Animal diarrheal Samples[74]
Halarcobacter bivalviorum	*Arcobacter bivalviorum*	F159-36	Arcobacter CAT broth + 2.5% NaCl	Marine Agar	Oyster exposed to Poble Nou Channel Water	Mussel from Ebro Delta, Spain[24]
Pseudarcobacter aquimarinus (Type)	*Arcobacter aquimarinus* (Type)	W63 ^{T} = CECT 8442 ^{T} = LMG 27923 ^{T}	Arcobacter CAT broth	Blood Agar	Seawater, Garraf beach, Catalonia, Spain[29]	Seawater, Garraf beach, Catalonia, Spain[29]
Pseudarcobacter aquimarinus	*Arcobacter aquimarinus*	F185-17	Arcobacter CAT broth	Blood Agar	Mussel exposed to Poble Nou Channel Water	Seawater, Garraf beach, Catalonia, Spain [29]
Malaciobacter molluscorum	*Arcobacter molluscorum*	F189-DG33	Arcobacter CAT broth + 2.5% NaCl	Marine Agar	Mussel exposed to Poble Nou Channel Water	Mussel, Ebro Delta, Spain[23]
Malaciobacter canalis (Type)	*Arcobacter canalis* (Type)	F138-33 ^{T} = CECT 8984 ^{T} = LMG 29148 ^{T}	Arcobacter CAT broth + 2.5% NaCl	Marine Agar	Oyster exposed to untreated sewage at Poble Nou Canal, Spain [2]	Oyster exposed to untreated sewage at Poble Nou Canal, Spain [2]
Malaciobacter canalis	*Arcobacter canalis*	F167F33	Arcobacter CAT broth + 2.5% NaCl	Marine Agar	Alfacs Bay Mussel	Oyster exposed to untreated sewage at Poble Nou Canal, Spain [2]
Malaciobacter marinus	*Arcobacter marinus*	W132-33	Arcobacter CAT broth + 2.5% NaCl	Marine Agar	Alfacs Bay Water	Seawater, Dokdo Island, Korea[75]
Malaciobacter halophilus	*Arcobacter halophilus*	F166-43	Arcobacter CAT broth + 2.5% NaCl	Marine Agar	Oyster exposed to Poble Nou Channel Water	Water from Hypersaline lagoon, HawaiianIslands[76]

Table 1. List of *Arcobacter*-like strains (origin, isolation source, first report) used for the development of an improved enrichment broth for the isolation of *Arcobacter*-like spp.

Artificial seawater (ASW) preparation. Artificial seawater (ASW) was prepared following the sea salt manufacturer's instructions (Aqua Medic MeerSalz, by AB Aqua Medic GmbH, Germany) by adding 31.4 gm per litre of distilled water, pH 8 ± 0.2, and salinity 34‰. All the media requiring seawater were prepared by using this ASW.

Growth monitoring. Growth was assessed by optical density (OD) readings at 420 nm, by means of a photometer (Model D-100, Dinko instruments, Spain). A preliminary OD vs. colony forming unit (CFUs) in marine agar, was performed with *M. canalis* to evaluate its correlation. The mean value was calculated considering the CFU counts obtained from triplicates of each plate and of every dilution. An OD vs. CFU correlation was made for all the three OD value (0.4, 0.6 and 0.8) tested.

Preparation of oyster matrices for media supplementation. Two types of oyster matrices were prepared as the organic nutrient source and were supplemented into the conventional broth media, as explained below.

Oyster homogenate (OH). Two kilograms (2 kg) of depurated oysters (*Crassostrea gigas*) produced locally in the Ebro Delta bays (Tarragona, Spain) were purchased from a commercial store. The oysters (n = 30) were of commercial size, ranging from 65 to 97 cm in shell length. They were rinsed and cleaned externally before opening. The oyster flesh (299 g) with its inter-valval liquid was collected in a sterile beaker. Then the samples were homogenized with a sterile kitchen blender. The homogenized oyster flesh was used in the preparation of the different kinds of broth used in the experiments.

Lyophilized oyster (LO). The Pacific oysters (*C. gigas*) were obtained from Ebro Delta, Spain and dry mass of lyophilized oysters, was prepared by freeze drying flesh and inter-valval liquid of oysters in a lyophiliser. The dry mass was then ground to a fine powder. The fine powder obtained after grinding was used as a supplement in the different experimental broth media.

Media preparation. *Broth media preparation.* Five groups of different broth media were prepared. These groups were based on using three conventional broth media with or without oyster supplementation (OH or LO) and prepared using different salt compositions (NaCl/ Artificial seawater [ASW]) and strengths (50% ASW or 100% ASW). The pH and salinity of each broth was measured. The experiment was done in duplicate.

Broth media	Broth #	Broth media name	Broth media abbreviation	Salinity (ppt)	pH
Conventional Broth Media	1	Arcobacter Broth	AB	7.6	7.06
	2	Marine Broth	MB	25.3	7.56
	3	Heart Infusion	HI	7.3	7.53
	4	Arcobacter Broth + 2.5% NaCl	ABNC	27.8	7.10
	5	Arcobacter Broth + 50% Artificial Sea Water (ASW)	ABSW	16.9	7.03
	6	Arcobacter Broth + 100% ASW	ABSW100	31.5	7.55
Conventional Broth Media + Homogenized Oyster	7	Arcobacter Broth + Oyster Homogenate (OH)	ABOH	4.2	7.13
	8	Arcobacter Broth + OH + 2.5% NaCl	ABOHN	25.7	7.04
	9	Arcobacter Broth + OH + 50% ASW	ABOHSW	16.8	7.13
	10	Arcobacter Broth + OH + 100% ASW	ABOHSW100	28.6	7.29
	11	Marine Broth + OH	MBOH	25.6	7.41
Homogenized Oyster Media	12	OH + d.H_2O	OH	1.2	7.09
	13	OH + 2.5% NaCl	OHNC	26.3	6.69
	14	OH + 50% ASW	OHSW	12.6	7.06
	15	OH + 100% ASW	OHSW100	25.8	7.35
Conventional Broth Media + Lyophilized Oyster	16	Arcobacter Broth + Lyophilized Oyster (LO)	ABL	5.6	7.08
	17	Arcobacter Broth + LO + 2.5% NaCl	ABLNC	28.1	6.88
	18	Arcobacter Broth + LO + 50% ASW	ABLSW	16.7	6.98
	19	Arcobacter Broth + LO + 100% ASW	ABLSW100	28.4	7.53
	20	Marine Broth + LO	MBL	25.7	7.56
Lyophilized Oyster Media	21	LO + d.H_2O	LO	2.2	6.95
	22	LO + d.H_2O + 2.5% NaCl	LONC	27.1	7.1
	23	LO + 50% ASW	LOSW	14.9	6.93
	24	LO + 100% ASW	LOSW100	27.4	7.25

Table 2. List of 24 broth media used in the experiment. The abbreviation given in the separate column for each broth media will be used in the figures/tables onwards.

Details of the five groups, making 24 types of broth media (Table 2) are given below;

1. **Conventional broth media**: The three conventional broth media used (Arcobacter broth, AB; Marine broth, MB and Heart Infusion, HI) were prepared according to the instructions from the manufacturer (Table 2). The Arcobacter broth was modified by adding 2.5% of NaCl[30] or by adding 50% and 100% artificial seawater (ASW).
2. **Enrichment broth media containing oyster homogenate**:

    2a. Conventional broth media supplemented with oyster homogenate (OH): This group of media were prepared by mixing half of the amount specified by the manufacturer instructions for conventional broth media (12g/L of Arcobacter broth _AB (Oxoid, UK) or 18.7 g/L of marine broth _MB (BD, Spain), with 25 g/L of homogenized oyster (HO). Additionally, three types of Arcobacter broth supplemented with homogenized oyster (ABOH) were prepared by adding 2.5% NaCl or 50% ASW or 100% ASW. Thus, this group contained five types of broth media (Broth# 7-11 of Table 2).

    2b. 2b. Oyster homogenate broth media: This media was prepared by adding 50 g/L of oyster homogenate into distilled water with no salt added or three different concentration (2.5% NaCl or 50% ASW or 100% ASW). It consisted of four types of broth media (Broth# 12-15 of Table 2).

3. **Enrichment broth media containing lyophilized oyster**:

    3a. Conventional broth media supplemented with lyophilized oyster: This group of media was prepared by supplementing 12 g/L Arcobacter broth or 18.7 g/L marine broth i.e. half amount of that given by manufacturer instruction for both conventional broth media, with 5 g/L lyophilized oyster. Arcobacter broth supplemented with LO (ABLO) were also prepared with 2.5% NaCl or 50% ASW or 100% ASW. This group contained five types of broth media (Broth# 16-20 of Table 2).

    3b. Lyophilized oyster broth media: This group of media consisted of four types of broth (Broth# 21-24 of Table 2) by adding 10 g/L of lyophilized oyster powder in to either distilled water or distilled water containing 2.5% NaCl or 50% or 100% ASW.

Experimental procedures. *Inoculum preparation.* Fresh colonies of the 14 *Arcobacter*-like species grown on marine and blood agar mentioned above (Table 1) were transferred to sterile marine broth and tryptic soy broth (TSB) tubes, respectively. The tubes were vortexed for 10 s and incubated in aerobic condition at 30 °C for 24 h to get the uniform fresh culture of the colonies in a liquid medium. The optical density at 420 nm was adjusted, with sterile marine broth, to OD value 0.1 for all the strains used in the study (Table 1).

Broth inoculation and incubation:. From the inoculum, 0.1 ml was transferred to the 9.9 ml pre-labelled broth tubes of different enrichment broth, vortexed and incubated in aerobic condition at 30 °C. The OD_{420nm} was measured for each strain at 0 h, 24 h and 48 h in different broth media (Table 2). Sterile broth of each formulation was used as control for the OD reading.

Inoculation on solid media:. After the incubation for 48 h, the growth of all the strains in the 24 types of enrichment broth was determined by culturing on marine agar, 100 µl of each enrichment broth. The plates were then incubated in aerobic condition at 30 °C for 48 h. Purity of the cultures was assessed, and relative growth was recorded as number of colonies following a scale from 0 to 4 (0 = No growth; 1 = 1–30 colonies; 2 = 31–150 colonies; 3 = 151–300 colonies; 4 = > 300 colonies).

Statistical analysis. The growth results are expressed in the terms of optical density (OD_{420nm}) for each strain tested in each enrichment broth used in this study. Values of the mean and standard deviation were calculated for duplicate OD_{420nm} values. Statistical analysis was performed in SPSS 21.0 (IBM SPSS Statistics, SPSS Inc., USA). Analysis of variance (ANOVA) and differences among the mean values were tested by Tukey's test. Significant difference was accepted for $p < 0.05$.

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Author contributions

F.U.R. performed experiments and interpreted the experimental data. F.U.R., K.B.A. and D.M.F. designed the experiments. N.S.M. performed the initial isolation of bacterial strains used in the study. F.U.R. and K.B.A drafted the manuscript. F.U.R and A.S. performed the statistical analysis. D.M.F., M.F.T. and M.J.F supervised the study. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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3.2 Study 2: Epidemiological study of unexplained mortality episodes in Pacific oyster (*Crassostrea gigas*) from the Ebro Delta, Spain: a possible polymicrobial etiology

(In-preparation)

RESULTS AND DISCUSSION

Summary

The Ebro Delta (Spain) is a large Pacific oyster (*Crassostrea gigas*) producing area, where several mortality events have been reported in both juvenile and adult stocks, attributed to the Ostreid herpesvirus type 1 (OsHV-1) and *Vibrio* spp., respectively. Previous studies on *C. gigas* microbiome during mortality events in the area and in other regions, revealed the presence of *Arcobacter*-like spp. The objective of this study was to screen both for the known pathogens and other potential ones to provide further insights into the *C. gigas* mortality events in the Ebro Delta. Thus, *C. gigas* samples were tested, over a two-year period (2018-2020), for OsHV-1, *Vibrio aestuarianus, Vibrio splendidus,* and *Arcobacter*-like spp. during 15 mortality episodes and compared with stocks not affected by mortality. Both molecular and cultivation techniques were implemented, including the development of a *Malaciobacter marinus* PCR.

It was found that Pacific oyster mortality events in the Ebro Delta were associated with polymicrobial infections with combinations of OsHV-1, *V. aestuarianus*, and *Malaciobacter marinus.* Specifically, OsHV-1 was detected in ten samples, *V. aestuarianus* in six, and *M. marinus* in all samples of the 15 mortality episodes studied. Mortalities in triploid adult oysters were significantly higher in warmer months (April-June) than in colder months (November-March), in fact, the highest prevalence of *V. aestuarianus* was registered in June 2019, associated with a mortality episode affecting 51% of adult oysters. Moreover, very important biological material was acquired from moribund oysters, including the first isolation of *V. aestuarianus* and several isolates of *Arcobacter*-like spp., which were essential for the further functional work performed in the thesis. The findings of this study could be useful for the development of management strategies to reduce the impact of these pathogens on oyster production in the Ebro Delta.

Epidemiological study of unexplained mortality episodes in Pacific oyster (*Crassostrea gigas*) from the Ebro Delta, Spain: a possible polymicrobial etiology

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Abstract

The Ebro Delta is the largest Pacific oyster (*Crassostrea gigas*) producing area along the Mediterranean coast of Spain, with 494 tons in 2021. Several mortality events have been reported in the delta in both juvenile and adult Pacific oysters, mainly attributed to the Ostreid herpesvirus type 1(OsHV-1) and *Vibrio* spp. respectively. Recent studies reported the presence of the latter pathogens along with *Arcobacter*-like spp. in the *C. gigas* microbiome, during mortality episodes in the Ebro Delta. The objective of this study is to screen the *C. gigas* mortality events in the Ebro Delta for the mentioned potential pathogens to provide further insights into such episodes. From November-2018 to June-2020, three classes of oyster samples (spat, juvenile and adult) from the Ebro Delta were collected during 15 mortality events and 4 contrasted non-mortality samples. The sampled oysters represented the ongoing commercial productions, including both normal diploid

(two sets of chromosomes) and faster growing triploid oysters three sets of chromosomes. The DNA extracted from the oyster tissues was screened by qPCR assays for OsHV-1, *Vibrio aestuarianus, Vibrio splendidus* and *Arcobacter*-like spp. Additionally, the *Vibrio* spp. and *Arcobacter*-like spp. were also screened by conventional culture media. The herpes virus OsHV-1 was detected in ten samples while *V. aestuarianus* was detected in six and isolated in five and all came from warm months (spring-summer) and mortality episodes. Even though *V. aestuarianus* was detected in all *C. gigas* age classes and ploidy groups its highest prevalence was in June-2019 (30%) associated with a mortality episode affecting 51% of the adult oysters. This is the first time that *V. aestuarianus* was isolated from *C. gigas* mortality events in the study area. Bacteria from the *V. splendidus* clade and *Arcobacter*-like spp. were detected and isolated in most samples irrespectively of age classes or ploidy groups of oysters and in all seasons, both during mortality and non-mortality episode. Characterization by genotyping, *rpoB* gene sequencing and phylogenetic analysis of a subset of *Arcobacter*-like spp., showed that *Malaciobacter marinus* was present in only during *C. gigas* mortality events but in all the samples This study indicates that the Pacific oyster mortality events in the Ebro Delta could be associated with polymicrobial infections produced by combinations of OsHV-1, *V. aestuarianus* and *M. marinus*.

Key words: Pathogenesis, Mortality, Ebro Delta, OsHV-1, *Vibrio, Arcobacter*, *Malaciobacter marinus*, *Crassostrea gigas*, Polymicrobial infections.

Introduction

About 10-12% of the world's population depends for their income on fisheries and aquaculture (Solomieu et al., 2015). Shellfish production is the main aquaculture sector in Spain (FAO 2022). Due to its rapid growth and adaptability to different environments, the Pacific oyster (*Crassostrea gigas*) has been introduced from Asia to numerous countries (De Lorgeril et al., 2018) and is prioritized for cultivation over other similar species (Prado-Alvarez et al., 2016). Thus, at present, *C. gigas*, is the main oyster species cultivated worldwide, accounting for 98% of the global oyster production (Guo, 2009; Azema et al., 2017). France is the top producer of Pacific oyster in the European Union (EU), with 45% of the total European aquaculture production in 2017 (EUMOFA, 2020). However, oyster production is highly vulnerable to disturbances by pollutants and infectious disease outbreaks (Morley, 2010; Raftos et al., 2014; Azema et al., 2017). Mass mortality events in Pacific oysters have been reported since the 1950s in most production areas, including Japan, the USA, Europe, New Zealand, and Australia (Pernet et al., 2016; De Lorgeril et al., 2020; Fleury et al., 2020). Different pathogenic agents (bacteria, viruses, and parasites) have been implicated in mortality events involving all oyster classes (Azema et al., 2016; Azema et al., 2017; Alfaro et al., 2019; Degremont et al., 2021). A current example of this problem is occurring in the largest shellfish production area on the Mediterranean coast of Spain located in the Ebro Delta, in the south of Catalonia, where the production of *C. gigas* has been affected by severe mortality episodes since 2006 (Carrasco et al., 2017; Fuhrmann et al., 2019). Since the start of the 21st century, repeated Pacific oyster mortalities have been observed in the Ebro Delta during springtime with little evidence to support etiological association with chemical contaminants from the agricultural practices around the bays, as previously considered (Carrasco et al., 2018). Decline in *C. gigas* production in the Ebro Delta area, has been attributed to the herpes virus OsHV-1 µvar,

mainly affecting early life stages (Roque et al., 2012, Carrasco et al., 2017). However, more recently, putative implication of *Vibrio aestuarianus* and/or *Vibrio splendidus* clade in adult oyster mortalities have been reported (Carrasco et al., 2018; Lasa et al., 2019).

In European countries such as France, Ireland and Spain, mass *C. gigas* mortalities have occurred in spat and juvenile oysters at water temperatures ranging from 16 °C to 24 °C (Garcia et al., 2011; Renault et al., 2014; Prado-Alvarez et al., 2016; Carrasco et al., 2017; Hick et al., 2018). The herpes virus OsHV-1 was reported for the first time in 2008 from juvenile oysters of the Ebro Delta harvesting area, (Roque et al., 2012) and since then has been regularly detected during episodes of juvenile mortality, coinciding with the water temperature passing the 16 °C threshold (Carrasco et al., 2017). Moreover, herpes viruses can persist in the host entering a latent phase, with their hosts becoming asymptomatic carriers; however, after several weeks or months it can re-activate and cause damage (Segarra et al., 2014). Fortunately, *C. gigas* can become resistant to OsHV-1 µvar, through selective breeding programs (Degremont et al., 2015; 2016), and with increased age (Degremont, 2013; Azema et al., 2016, Petton et al., 2019). While resistant strains of oysters are a useful mitigation tool, active disease surveillance and management strategies to better cope with the presence of the virus are essential for sustainable production (Carrasco at al., 2017; Cain at al., 2021).

The genus *Vibrio* is composed of ubiquitous aquatic bacteria, including diverse members of planktonic and animal-associated microbial communities (Le Roux et al., 2009; 2015; Foulon et al., 2016). Several species within this genus, such as *Vibrio alginolyticus*, *Vibrio anguillarum*, *Vibrio splendidus* biovar II, *Vibrio tubiashii*, and *Vibrio coralliilyticus*, have globally been associated with mass mortalities of juvenile bivalves in oyster hatcheries (Gomez-Leon et al., 2005; Kim et al., 2020).

Vibrio aestuarianus is an important bacterial pathogen responsible for summer mass mortalities in adult oysters (Goudenege et al., 2015). This species was described in 2008 associated with mortalities in *C. gigas* from France (Garnier et al., 2008), which is the main supplier of *C. gigas* spat and juveniles to the Ebro Delta production area. The presence of *V. aestuarianus* has been linked to mortality events since 2012 in pre-commercial and commercial-size *C. gigas* in France and Ireland (Azema et al., 2017). Despite the proximity with France, the origin of the Ebro Delta stock no mortality events of *C. gigas* associated to this pathogen had been reported in the Ebro Delta bays (Alfacs and Fangar) of the Spanish Mediterranean coast until May 2017, when a massive mortality event of commercial size oyster occurred, at a water temperature of 20 ºC and it was putatively attributed to *V. aestuarianus* (Carrasco et al., 2018).

V. splendidus has been described as affecting fish species such as turbot, *Scophthalmus maximus* (Gatesoupe et al., 1999), and associated with mortalities of early stages of different mollusk species such scallops i.e., *Pecten maximus* (Nicolas et al., 1996), *Patinopecten yessoensis* (Liu et al., 2013, and oyster species worldwide including *C. virginica* (Elston and Leibovitz, 1980) and *C. angulata* (Comps, 1988). Specifically, to this work, members of the *V. splendidus* clade have been associated with mortality outbreaks causing significant losses in the aquaculture industry worldwide of Pacific oyster *C. gigas* (Nasfi et al., 2015; Bruto et al., 2018, Lacoste et al., 2001; Le Roux et al., 2002). However, members of the *V. splendidus* clade are ubiquitous, frequently reported as common colonizers of marine and estuarine waters, and even sediments (Vezzulli et al., 2015). Accordingly, a large genetic and phenotypic plasticity has been observed within the species of *V. splendidus* (Le Roux et al., 2002; 2004), comprising both virulent (Lipp et al., 2001) and avirulent ones (Saulnier et al., 2010). Along those lines, in the shellfish producing areas of the Ebro Delta, members of the *V. splendidus* clade have been found in *C. gigas* samples from both

mortality and non-mortality episodes (Carrasco et al., 2018; Lasa et al., 2019). The polyphyletic nature of this clade and the lack of clear discrimination among the species by biochemical or molecular methods make it difficult to differentiate between virulent and non-virulent strains (Le Roux et al., 2004).

Arcobacter-like spp. are cosmopolitan in their nature and have been recovered from different hosts and environments, which make *Arcobacter* an atypical genus within the epsilon Proteobacteria (Debruyne et al., 2008; Collado and Figueras, 2011; Salas-Massó et al., 2018). The genus *Arcobacter* has been subdivided in seven genus and the species previously known to be potentially pathogenic to humans and animals i.e., *Arcobacter butzleri, Arcobacter cryaerophilus, Arcobacter skirrowii, Arcobacter cibarius, Arcobacter therius* and *Arcobacter porcinus* (Collado and Figueras, 2011; Ferreira et al., 2014; Van den Abeele et al., 2014; Figueras et al., 2014; Salas-Massó et al., 2016; 2018) are now included in the genus *Aliarcobacter* (Pérez-Cataluña et al., 2018). The *Arcobacter*-like spp. are abundant in marine environment and frequently isolated from seafood (Collado et al., 2009a, b; Collado and Figueras, 2011; Rathlavath et al., 2017; Zhang et al., 2019; Mudadu et al., 2021) such as shellfish like abalone (Mizutani et al., 2019), mussels, clams and oysters (Collado et al., 2009a, b; Figueras et al., 2011; Levican et al., 2012; Collado et al., 2014; Levican et al., 2015; Mottola et al., 2016; Salas-Massó et al., 2016; 2018), copepods (Gugliandolo et al., 2008) and lobsters (Meziti et al., 2012). However, their role in the marine environment and as potential pathogen of aquatic animals is still unknown. Remarkably, the species *Arcobacter mytili* (now consider *Malaciobacter mytili*), was discovered as a new species from mussels and brackish water samples from Catalonia, Spain (Collado et al., 2009a, b; Pérez-Cataluña et al., 2018), and it was later reported in USA to cause the first notified case of symptomatic human bacteremia in a 65-year-old worker of a crab farm, suggesting the pathogenic

nature of this bacteria (Vasiljevic et al., 2019). Although, *Arcobacter*-like spp. are reported in several studies to be abundantly found during mortality episodes in *C. gigas,* their role in pathogenesis and their virulence potential towards the oysters are still unknown (Green et al., 2019; Lasa et al., 2019).

Lasa et al. (2019) compared the microbiota community structure of *C. gigas* in healthy oyster vs oysters from mass mortality episodes across European shellfish producing zones and revealed that the diseased oysters showed a disrupted community structure, with a lower diversity. The latter were dominated by bacteria of the genera *Vibrio* and *Arcobacter* in the case of the oysters infected *V. aestuarianus*. The authors concluded that those bacteria should be targeted in future studies to provide additional information about those polymicrobial infections in *C. gigas* (Lasa et al., 2019). An infection by OsHV-1 followed by polymicrobial bacteremia has been found to be an important cause of severe mortalities in *C. gigas* (Petton et al., 2015; 2019; 2021). Thus, there is often an incomplete understanding of diseases in which: i) a conserved consortium of micro-organisms creates synergy that induces pathogenesis, or ii) of diseases involving pathogens that cause immune deficiency followed by secondary infections, or iii) diseases that are influenced by a series of host and environmental factors (Gomez-Leon et al., 2005; Wang et al., 2010; Russell et al., 2017; De Lorgeril et al., 2018).

Taking all the above into account, the objective of the present study was to investigate if polymicrobial infections of *C. gigas* could be associated with the mortality episodes in the Ebro Delta. To that aim, the prevalence of the known viral and bacterial pathogens, OsHV-1, *V. aestuarianus, V. splendidus* clade were monitored during mortality episodes and in contrasted situations of non- mortalities. Moreover, the presence, relative abundance and potential virulence

of *Arcobacter*-like bacteria was also assessed, to increase the knowledge of their role in the mortality episodes.

Materials and Methods

Oyster samples collection

The sampling of the Pacific oysters (Supplementary table 1 was conducted from November 2018 to June 2020 in the aquaculture production sites of the two bays, Alfacs and Fangar, of the Ebro Delta, Spain (Figure 1). The seawater temperature, salinity and oxygen concentration were measured using a SeaBird 19plus (Sea-Bird Scientific, Bellevue, Washington, USA) at the site of collection and sampling coordinates were recorded. Additionally, the registered environmental data were retrieved from November 2018 to June 2020 from the shellfish growing areas Monitoring program of IRTA (https://www.marinemonitoring.org/informes/). Regarding the production area, most samples were collected in mortality episodes in Fangar Bay (14 from mortality episodes, and 2 non-mortality samples out of total 19 samples) which is the main site for oyster growing in the Delta (Supplementary tables 1 & 2). Samples collected, during each mortality or non-mortality sampling, consisted of an entire shellfish rope, (one for sampling point) from the bateas (rafts); standard ropes were 5-meter-long and held approximately 90-100 animals. Samples were transported to IRTA, La Ràpita, (Spain), where cumulative mortality was calculated (Mortality % = Total number of dead individuals/Total number of individuals in the sample x100) and the size of the animals (mm) was longitudinally measured. The juvenile oysters from IRTA's experimental hatchery, were produced under controlled conditions to avoid OsHV-1 infection, and they were used as internal controls, since they were always in the study area.

The oyster tissues were collected aseptically for DNA extraction, histology, and microbiological culturing, see below.

Sampling strategy

Most of the oyster samples i.e., 15/19 (79%) were collected during mortality episodes, while additional contrasted non-mortality ones, 4/19 (21%), of similar characteristics (size) were also collected, whenever available, to compare the environmental parameters and the microbiology findings of the mortality and non-mortality samples (Supplementary table1). When more than one sampling was conducted on the same day in one mortality episode from different collection points, with different reports of mortalities, these samples were labeled as A, B, and C to differentiate them. Oysters were marked as dead when their open valves didn't close upon touching, if the oysters loosely closed their valves, they were considered sick, while the oyster which could completely close their valve was considered healthy ones.

From each sample taken during a mortality episode, between 20 and 30 oysters were processed for microbiology and molecular biology (Supplementary table 2). Pathogen prevalence (%) was calculated as: Number of positive samples/ Number of total samples x 100. The oysters were grouped based on their shell height (longitudinal) into spat (<30 mm), juvenile (30-49 mm) and adult (>50 mm) according to Melo et al. (2020). The ploidy, according to the producer's information, (D = diploid/ T = triploid) and origin (A = Alfacs/ F = Fangar) of the oysters was also recorded (Supplementary table 2).

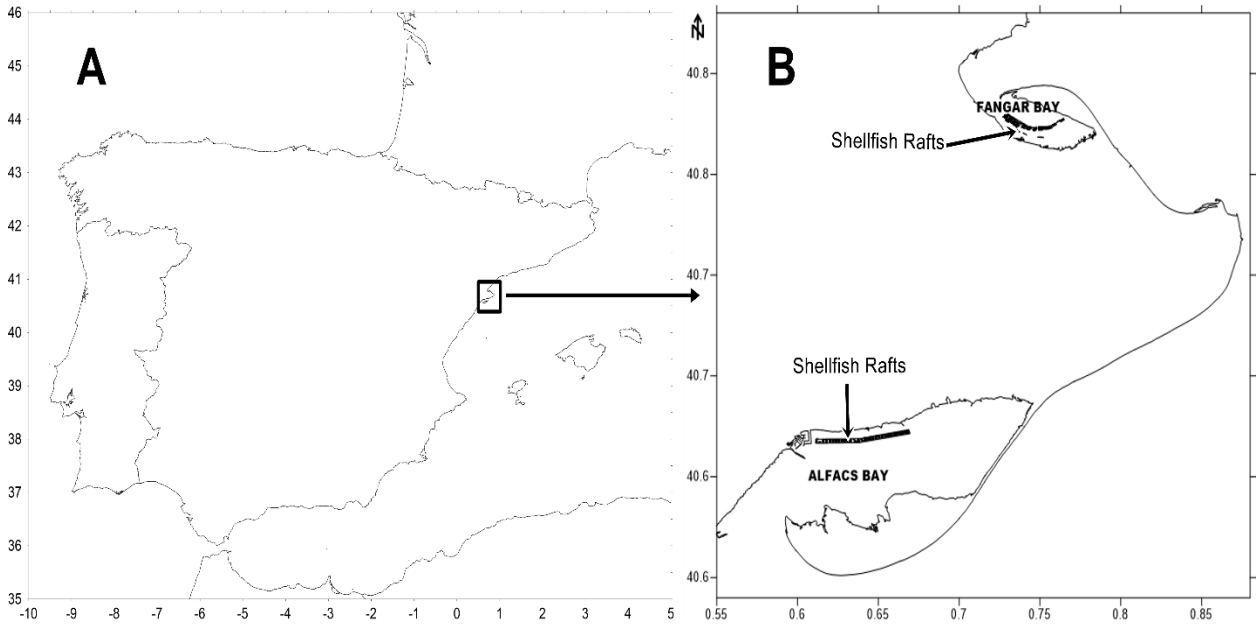


Figure 1: Location of the Alfacs and Fangar Bays in the Ebro Delta, Catalonia, Spain, from which oysters were harvested. In B it indicates the location of shellfish rafts from where the samples were collected.

DNA extraction and qPCR analysis

On arrival from the field oysters were washed with tap water before opening with a sterile knife. Each oyster was transversally cut and small pieces of all major organ tissues of 100 mg in total (i.e., mantle, gonad, digestive gland, adductor muscle and gills), were collected into 2 mL tubes, for DNA extraction, containing approximately 50 mg of 0.5 mm diameter zirconium glass beads (Biospec Products, Bartlesville USA). Tissues were homogenized in a BeadBeater-8 (Biospec Products), using a pulse of 40 seconds at full speed. After homogenization, the DNA was extracted and purified using the QIAGEN DNeasy® Blood & Tissue Kit (QIAGEN, Ref. #69506, Hilden, Germany). For determining DNA concentration and evaluation of purity, a NanoDrop 2000

(Thermofisher, Wilmington, USA) was used to confirm all samples had an $A_{260/280}$ ratio between 1.8- 2.0.

All diagnostic PCR reactions were performed in a 20 or 25 µL volume containing 0.5 µM of each primer (forward and reverse) needed for the detection of the pathogen under investigation. The thermal profiles used for programming the thermal cycler (Master Cycler Nexus Gradient, Eppendorf) were those published (Supplementary table 3) for the detection of OsHV-1 (Webb et al., 2007), *Arcobacter*-like spp. (Salas-Massó et al., 2019) and *Malaciobacter marinus* (developed in the current study which method is described below). All reactions included 100 ng of oyster tissue DNA. While in the qPCR assays for *Vibrio aestuarianus* and *V. splendidus* clade, 50 ng of oyster tissue DNA were included in a 25 µL reaction following the protocol outlined in Saulnier et al. (2017), and the reactions were performed in an ABI 3700 (Life Technologies). These qPCR assays were used as qualitative assays to screen for the positive and negative oyster tissue DNA collected during the study period and no standard curve was used to correlate with the colony count resulted from the growth of these tissue on agar media.

Bacterial culturing

After collecting the tissue sample for DNA extraction, the remaining half oysters along with inter-valval liquid were aseptically collected into pools of 5 oysters for bacterial culturing where all organ tissues (i.e., mantle, gonad, adductor muscle, digestive gland, and gills), were represented. Each pooled sample was weighed and homogenized with equal amount (w/v) of PBS (Phosphate Buffer Saline) for 2 minutes in a stomacher with double stomacher bags, the inner one including the filter cloth. The tissue homogenates were collected into sterile falcon tubes for further dilutions and plating. These tissue homogenates were used to screen and isolate *Vibrio aestuarianus, V. splendidus* clade and *Arcobacter*-like spp. by the methods specified below.

a. Vibrio aestuarianus and *V. splendidus* isolation

The pooled tissue homogenates (1 mL) from each sampling episode, were serially diluted tenfold (up to 10^{-2}) in sterile PBS and 100 µL aliquot of each of the pooled dilutions were spread onto marine agar (MA) plates, which were aerobically incubated at 23 ºC for 48 hours, before counting and isolation of individual presumptive colonies of type compatible with both Vibrios. For *V. splendidus* clade morphotypes (beige color round colonies, 1-3 mm in size, having smooth margins as described by Beaz-Hidalgo et al. (2009) were selected and for *V. aestuarianus* (opaque, < 1mm in size, smooth colonies with round margins) as described by Garnier et al. (2008). The colony morphotypes compatible with both *Vibrio* species, were distinctly recorded for both mortality and non-mortality episode samples, as proxy of their relative abundance. Four representative colonies of each selected morphotype were sub-cultured on MA and incubated under the same conditions to obtain pure cultures. Colony morphologies were recorded and coded; a loopful of each colony type was transferred to 180 µL of ATL buffer (QIAGEN, Ref.# 69506, Hilden, Germany) for bacterial genomic DNA extraction and pure cultures were preserved at -80 ºC in marine broth (PanReac AppliChem, C806142, Barcelona, Spain) supplemented with 20% (v/v) glycerol (Howard 1956).

b. *Arcobacter*-like spp. isolation

To isolate the *Arcobacter*-like species from the tissue homogenates, an enrichment step was performed in Arcobacter broth + CAT + 50% artificial seawater (Rahman et al., 2020) The incubation in CAT antibiotic mixture (C.A.T selective supplement: Cefoperazone-Amphotericin B-Teicoplanin) Oxoid™, SR0174E, Hampshire, UK] is to favor *Arcobacter* growth. From each oyster homogenate, 1 mL was transferred to 9 mL of the enrichment broth. The tubes were vortexed for 10 seconds and incubated aerobically at 23 ºC in static conditions for 48 hours.

Subsequently, the tubes were vortexed for 10 seconds and 100 µl of the enrichment broth were transferred onto a 0.45 µm nitrocellulose membrane filters of 47 mm diameter (Millipore, Ref # HAWP04700, Tullagreen, Ireland) placed upon a MA plate (Scharlab S.L., E08181 Sentmenat, Spain) for non-*Arcobacter*-like cells size exclusion. The plates were incubated for 30 minutes at room temperature to allow smaller bacteria such as *Arcobacter*-like spp. to pass through. Afterwards, the filter papers were removed, and the plates were incubated aerobically for 48 hours at 23 ºC. As for Vibrios, after incubation, colony counts of the morphotypes compatible with *Arcobacter*-like spp. were recorded. From each MA plate, four similar colonies of *Arcobacter*-like spp. (i.e., small <1 mm, transparent/translucent, beige, or off-white colonies) were sub-cultured on MA media and incubated for 48 hours at 23 ºC. Samples of cultures were processed for DNA extraction and colony preservation in glycerol as stated above (a).

Bacterial DNA extraction and quantitative PCR (qPCR)

The bacterial genomic DNA was extracted using a DNeasy® Blood & Tissue kit (QIAGEN, Ref.# 69506, Hilden, Germany). The DNA was quantified, and purity confirmed using a NanoDrop 2000 spectrophotometer (Thermofisher, Wilmington, USA). An aliquot of 5 ng/µL DNA concentration was prepared from each isolate. Then, for *Vibrio* duplex qPCR (Saulnier et al., 2017) and genus-specific 23S qPCR assays for *Arcobacter*-like spp. (Salas-Massó et al., 2019) were performed in duplicate for all isolates (Applied Biosystems®, Ref.# 4309155, Woolston Warrington, UK). Each reaction included 5 µL (5 ng/µL) and 1 µL (5 ng/µL) of the *Vibrio* spp. or *Arcobacter*-like spp. colony DNA preparation, in 25 µL and 20 µL reaction assay, respectively. For both of these assays the results are interpreted as positive or negative depending on: a) in the case of the Vibrio duplex assays the binding of the fluorescent probe that resulted in a positive signal; or b) in the case of the 23S Arcobacter-like qPCR the presence of a melt curve that could be compared to that of the

positive control samples for confirmation of a positive amplification. Standard curves were not included as these assays were used for purposes of identification only.

Genotyping of *Arcobacter*-like spp. by ERIC-PCR

To reduce the number of isolates to be tested and avoid working with multiple clones, genotyping of all *Arcobacter*-like isolates was carried out by a 50 µL Enterobacterial Repetitive Intergenic Consensus PCR (ERIC–PCR) reaction using 1R and ERIC 2 primers (Supplementary table 1) (Versalovic et al.,1991). A 2% agarose gel was run with 5 µL of amplified ERIC-PCR products from each sample/isolate at 100 volts for 2 hours along with Invitrogen 1kb plus molecular weight standard (Invitrogen^{TM}, Ref.# 10787-018, Carlsbad, USA). All ERIC-PCR banding patterns were visually compared with each other among the isolates and with the 14 original *Arcobacter*-like spp. type strains of marine origin (Table 1). The isolates were marked as belonging to the same genetic cohort if their ERIC-PCR banding pattern exhibit differences of one or more bands in the pattern (Houf et al., 2002; Salas-Massó et al., 2016).

Identification of *Arcobacter*-like isolates

To identify the species of *Arcobacter*-like isolates, the *rpoB* gene was amplified for one representative of each ERIC-PCR genotype (Salas-Massó et al., 2016; Collado et al., 2009). A 50 µL PCR reaction was performed containing 2 µL bacterial DNA (5 ng/µL) and 48 µL PCR mix. The PCR mix for *rpo B* amplification consisted of 5 µL reaction buffer (10X), 2 µL of $MgCl_2$ (50 mM), 4 µL of dNTP's (10 mM), 0.2 µL of Taq DNA polymerase (5 U/µL); (Invitrogen, Ref.# 10342-020, Carlsbad, USA), 1 µL forward primer and 1 µL reverse primer (20 µM each), and 34.8 µL of sterile ultra-pure distilled water. The amplified product was analyzed on a 1.5% agarose gel. Purification of the PCR product was achieved using the QIAquick® PCR purification kit (QIAGEN, Ref.# 28104, Hilden, Germany). Sequencing of the *rpoB* gene was done commercially

(Sistemas Genómicos, Valencia, Spain) using the same primers used for amplification. After obtaining the forward and reverse reads of each amplicon from the *Arcobacter*-like isolates, the consensus sequences of *rpoB* gene were compiled for each isolate using BioEdit 7.2.5 (BioEdit Sequence Alignment Editor, Hall, T.A. 1999). Presumptive identification of each strain by *rpoB* sequencing was done using the nucleotide BLAST search analysis tool (Nucleotide BLAST: Search nucleotide databases using a nucleotide query (nih.gov)) to match the *rpoB* gene consensus sequence with known *Arcobacter*-like spp. in the NCBI database. The BLAST hits with high similarity scoring were downloaded in FASTA format from the NCBI database and an alignment was created, along with the newly obtained *rpoB* sequences from *Arcobacter*-like isolates, using Clustal W multiple alignment tool in BioEdit 7.2.5 (BioEdit Sequence Alignment Editor, Hall, T.A. 1999). Then, all aligned sequences were trimmed to equal length (425 bp) for phylogenetic analysis. The phylogenetic tree was constructed by Neighbor-Joining (NJ) algorithms including 1000 boot strap replicates using the Kimura 2-parameter model in MEGA 6 software (MEGA, Tamura et al., 2013).

After phylogenetic characterization of the *Arcobacter*-like isolates by *rpoB* gene sequencing, the proportion of *Malaciobacter marinus* and *Arcobacter*-like spp. (other than *M. marinus*) was calculated to find their relative proportions in the study period. This proportion (%) of *M. marinus* and *Arcobacter*-like spp. other than *M. marinus* was calculated from different mortality and non-mortality samples of Pacific oysters by; Number of isolates of each bacteria / total number of isolates x 100.

Primer designing for *Malaciobacter marinus*-specific PCR

For the development of the *M. marinus*-specific PCR, 279 sequences of the *rpoB* gene from multiple strains of 38 species of other *Arcobacter*-like type spp./strains from the NCBI database

were aligned for identification of consensus regions. This screening alignment in BioEdit 7 (Hall 1999) revealed the existence of unique sites for primer design for *M. marinus*. A set of forward and reverse primers Mma rpoB-F and Mma rpoB-R (Supplementary table 3) that amplified a 135 bp sequence of the *rpoB* gene were designed for use. The optimization of the PCR assay using these primers was performed with a comparison of 15 different *Arcobacter*-like spp./strains as controls (Table 1). The 20 µl PCR mix for each sample included 2 µl of buffer (10X) (Invitrogen, CA), 0.6 µl of $MgCl_2$ (50 mM), 1.2 µl of dNTP's (10 mM), 0.6 µl of each Mma rpoB-F (20 µM) and Mma rpoB-R (20 µM) primer, 0.1 µl of *Taq* DNA Polymerase (5 U/µl) (Ref# 10342-020, Invitrogen, Carlsbad, CA), and 13.90 µl of sterile ultrapure distilled water (Invitrogen, Ref.# 10977-035, Paisley, UK). Then 1 µl of the DNA sample (5 ng/µl) was added to the reaction mixture. All amplifications were performed in an Eppendorf Master cycler Nexus Gradient thermocycler (Eppendorf, Hamburg, Germany) with reaction conditions according to the following: 95 °C for 3 min followed by 40 cycles of denaturation at 95 °C for 30 s, primer annealing at 66.3 °C for 20 s and chain extension at 72 °C for 30 s with a final extension step at 72 °C for 5 min. This annealing temperature of 66.3 ºC was a critical condition for avoiding amplification of *M. canalis* DNA, which is highly similar in the *rpoB* region. The amplified product was visualized on 2.5% agarose gel for 45 minutes using TAE buffer.

Development of quantitative PCR (qPCR) assay for *M. marinus*

The above-described PCR was adapted as a quantitative PCR assay to avoid the need for running gels. Additionally, qPCR performed using SYBER Green, or similar intercalating dyes, enables inspection of melt curves to differentiate false amplifications from true positives, since melt curve profiles can be used as a proxy for the DNA sequence that has been amplified (Andree et al., 2011). For this, reactions were performed using 20 µL reactions in 96 well plate in triplicate using the

7300 real-time PCR system (Applied Biosystems®, Marsiling, Singapore). Initially, a 5 ng/µL of genomic DNA from three *M. marinus* strains IRTA-19-131, IRTA-19-132 (Isolated during this study) and strain F165-33 (isolated by Salas-Massó et al., 2016) were 10-fold serially diluted in sterile ultrapure distilled water (Invitrogen, Ref.# 10977-035, Paisley, UK) down to a concentration of 0.5 fg/µL (10^{-7}) gDNA of *M. marinus*. Each 20 µL reaction contained, 10 µL of SYBR green PCR master mix (2X) (Applied Biosystems®, Ref.# 4309155, Woolston Warrington, UK), 0.5 µL of each Mm-rpoB forward and reverse primer (20 µM each) and 8 µL of sterile ultrapure distilled water (Invitrogen, Ref.# 10977-035, Paisley, UK) with the addition of 1 µL of *M. marinus* gDNA each serially diluted 10-fold (5 ng/µL to 0.5 fg/µL). Initially, the system was heated at 95 °C for 10 min followed by 40 cycles of denaturation at 94 °C for 40 seconds, primer annealing at 66.3 °C for 30 s and primer extension at 72 °C for 30 s. A melt curve analysis step was included at the end of the PCR reactions to verify that only the intended amplicon of *M. marinus* was produced. The qPCR essay efficiency % (E) = $-1+10^{(-1/\text{slope})}$, limit of detection (LoD) was calculated according to the method described before (Hausdorf et al., 2013). Additionally, aliquots of 50 ng/µL of *C. gigas* tissue DNA was spiked with *M. marinus* gDNA in the final concentration given above (5ng/ µL to 0.5 fg/µL) to check for any reduced efficiency in amplification or signal loss from *M. marinus* DNA due to the presence of oyster tissue DNA.

Moreover, to find out how many CFU's/mL of the *M. marinus* can be detected by this qPCR assay, a bacterial suspension of 0.812 OD_{420nm} corresponding to 10^8 CFU/mL of *M. marinus* was prepared in sterilized PBS from fresh overnight cultures and diluted down to 10^0 by 10-fold serial dilution. From each dilution 100 µL of the suspension was inoculated by spreading on marine agar plates in triplicates to calculate the CFU's/mL of the suspension. Another 1 ml of the suspension from each dilution was centrifuged at 8,000 rpm for 20 minutes to collect the bacterial cells and then

the supernatant was discarded. Then 180 µL of ATL buffer and 20 µL of the proteinase K (Qiagen, Hilden Germany) was added to each dilution (10^0 to 10^8) and the tubes were vortexed thoroughly for 30 seconds to resuspend the cell pellet. The DNA was extracted using DNeasy® Blood & Tissue kit (QIAGEN, Ref.# 69506, Hilden, Germany) following the protocol of the manufacturer. The quality and quantity of the genomic DNA obtained were quantified by NanoDrop 2000 (Thermofisher, Wilmington, USA). The quantitative *M. marinus* Mma-rpoB qPCR assay was carried out for all these dilutions according to the conditions described above and LoD was calculated as discussed above.

Table 1: List of strains used as a control in the *Malaciobacter marinus* qPCR development.

S. No.	**Strain name**	**Strain code in URV collection**	**Isolation source**
1	*Aliiarcobacter butzleri* (Type)	LMG 10828^T	Man with diarrhoea, faeces (USA, 1990)
2	*Aliiarcobacter cryaerophilus*	F169-1G17	Mussel exposed to Poble Nou channel water, Ebro Delta, Spain
3	*Aliiarcobacter hispanicus*	F164-18	Oyster, Alfacs Bay, Spain
4	*Aliiarcobacter skirrowii*	W125-41	Poble Nou Channel water
5	*Arcobacter nitrofigilis*	W111-35	Poble Nou Channel water
6	*Halarcobacter bivalviorum*	F159-36	Oyster exposed to Poble Nou Channel water
7	*Halarcobacter mediterraneus*	W143-33	Poble Nou Channel water
8	*Malaciobacter canalis*	F167F33	Mussel, Alfacs Bay

9	*Malaciobacter canalis* (Type)	F138-33 ^{T} = CECT 8984 ^{T}	Oyster exposed to Poble Nou Channel, Spain
10	*Malaciobacter halophilus*	F166-43	Oyster exposed to Poble Nou Channel water
11	*Malaciobacter marinus*	F165-33	Oyster, Alfacs bay
12	*Malaciobacter molluscorum*	F189-DG33	Mussel exposed to Poble Nou Channel water
13	*Pseudarcobacter aquimarinus*	F185-17	Mussel exposed to Poble Nou Channel water
14	*Pseudarcobacter aquimarinus* (Type)	W63 ^{T} = CECT 8442 ^{T}	Seawater, Garraf beach, Catalonia, Spain
15	*Pseudoarcobacter ellisii*	F140-17	Clam, Alfacs bay, Spain

Amplification of putative virulence genes in *Arcobacter*-like isolates

All representative genotypes of *Arcobacter*-like isolates were screened for the presence/absence of nine putative virulence genes i.e., *cadF, hecA, hecB, ciaB, cj1349, irgA, pldA, tlyA* and *mviN*. These virulence genes are reported from several terrestrial, marine and food origin *Arcobacter*-like spp. e.g., *A. butzleri, A. skirrowii* and *A. therius* and several other similar species (Collado and Figueras, 2011; Douidah et al., 2012; Collado et al., 2014). A 50 µL PCR reaction was performed for each sample using the specific primer set for each gene (Supplementary table 3) (Douidah et al., 2012; Collado et al., 2014). *Aliiarcobacter butzleri* LMG 10828^{T} (previously known as *Arcobacter butzleri*, Peréz-Cataluña et al., 2018) DNA was used as a positive control which harbors in its genome all the putative virulence genes mentioned (Douidah et al., 2012). The primers were divided into two sets A and B, for the difference in the quantity of $MgCl_2$ used in the PCR mix and annealing temperatures required for primer hybridization. The 50 µL reaction of

both primer groups consisted of 2 µL of DNA extract (50 ng/µL), 1.5 U of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA), 0.2 mM each deoxyribonucleotide triphosphate (dNTP's), 50 pmol of each primer set, 1X PCR buffer (Invitrogen, CA). For the group A primer sets (*ciaB, cj1349, hecA, irgA* and *mviN*) 1.3 mM $MgCl_2$ was used while for the group B primer sets (*cadF*, *hecB, pldA* and *tlyA*) 1.5 mM $MgCl_2$ was used for each separate PCR reaction for each gene. The PCR reaction conditions for all genes were 94 °C for 3 min samples heating followed by 32 cycles of denaturation at 94 °C for 45 s, primer annealing at 56 °C for 45 s for group A primer sets, while 55 °C for 45 s for group B primer sets and primer extension was at 72 °C for 45 s, then ending with a final extension step at 72 °C for 3 min.

The amplified PCR products of each gene were visualized by agarose gel electrophoresis using 1.5% agarose in 1X Tris-acetate-EDTA (TAE) buffer at 100 V for 60 min. A gel documentation system (GeneFlash, Syngene Bioimaging, Cambridge, UK) was used for visualization and interpretation.

Statistical analysis

The mean and standard deviation of the PCR assay results were calculated in Microsoft excel. A Pearson correlation was calculated to examine for any correlation between mortalities and the viral or bacterial pathogens screened and isolated from Pacific oyster in both mortality and non-mortality episodes. Differences were considered significant at values of $P < 0.05$. Statistical data analysis was carried out in SPSS (version 21.0) for Windows (IBM Corp, Armonk, NY, USA).

Results

During the two years study period, the average seawater temperature in Alfacs Bay was 17.9 °C (min.: 8.3-max.: 29.3 °C); and 17.2 °C (min.: 7-max.: 28.3 °C) in Fangar Bay. The average salinity

(S_P) in Alfacs Bay was 35.2 (min.: 30.6-37.3); 31.9 (min.: 14.1-max.: 38) in Fangar Bay. Average oxygen concentration in Alfacs Bay was 6.9 mg/L (min.: 5-max.: 8.9 mg/L); 7.5 mg/L (min.: 5.2-max.: 9.9 mg/L) in Fangar Bay (data not shown).

The recorded cumulative mortality of the samples collected from Ebro Delta's Alfacs and Fangar Bays varied among different months, bays, animal sizes, ploidy (supplementary tables 1 and 2), and environmental conditions (mainly temperature where the mortality increased when temperature increased from 15 °C). Based on unpublished historical data, the samples that showed a cumulative mortality of 7% or more were considered as "mortality episodes", while below this level were considered "non-mortality". As for age groups, the samples included all oysters age groups, i.e., spat (<30 mm), juvenile (30-49 mm) and adults (>50 mm), although adult *C. gigas* were particularly targeted (15/19) due to the increasing frequency of episodes in this age group in the Delta. Moreover, the adult oyster mortality episodes were only studied in Fangar Bay (Figure 2), as mortality was not reported from Alfacs Bay in adult oysters during the study period. Considering ploidy, the highest cumulative mortality in adult oysters was reported in triploid adult oysters (68%) in 2020/Jun/04-A, while the parallel highest mortality in diploid adult oysters (2020/Jun/17-B) registered was 31% which was much lower mortality that observed in triploid oyster, mentioned earlier (Supplementary table 1 & 2). In juveniles, the highest mortality was also observed in Fangar Bay, with 51% cumulative mortality in 2019/Mar/28-C diploid oyster samples compared to 17% of 2019/Mar/28-B (Supplementary table 1) samples from Alfacs Bay in commercial juvenile diploid oysters, while IRTA's diploid stock did not register mortality at the time. The only mortality of spat was registered in diploid oysters in the 2019/Jun/27 samples, from Fangar Bay, during summer (Supplementary table 1 and Figure 2), when a 33 % cumulative mortality was reported. Overall, the mortality samples split almost even between diploid and

triploid oysters, with 8/19 (42.0%) and 7/19 (37.0%), respectively. However, four of the eight mortality episodes in diploids were adult as occurred in all seven triploid oyster mortality events (Supplementary table 1 and Figure 2). In contrast, all four of 19 (21%) non-mortality samples originated from diploid animals. The mortalities in triploid adult oysters were significantly ($p<0.05$) higher in warmer months (April-June), when the temperature increased to 15 ºC or above, than in colder months (November-March) (Figure2). There was no significant ($p>0.05$) correlation of salinity with oyster mortality for any age group. Since only one sampling episode was recorded for spat and juvenile oysters, no statistical analysis could be performed regarding size due to the limited data.

Macroscopically, there was no visible sign of pathogenesis e.g., pustules, abscesses, or coloration on oyster tissues in any of the oysters sampled, both alive and moribund.

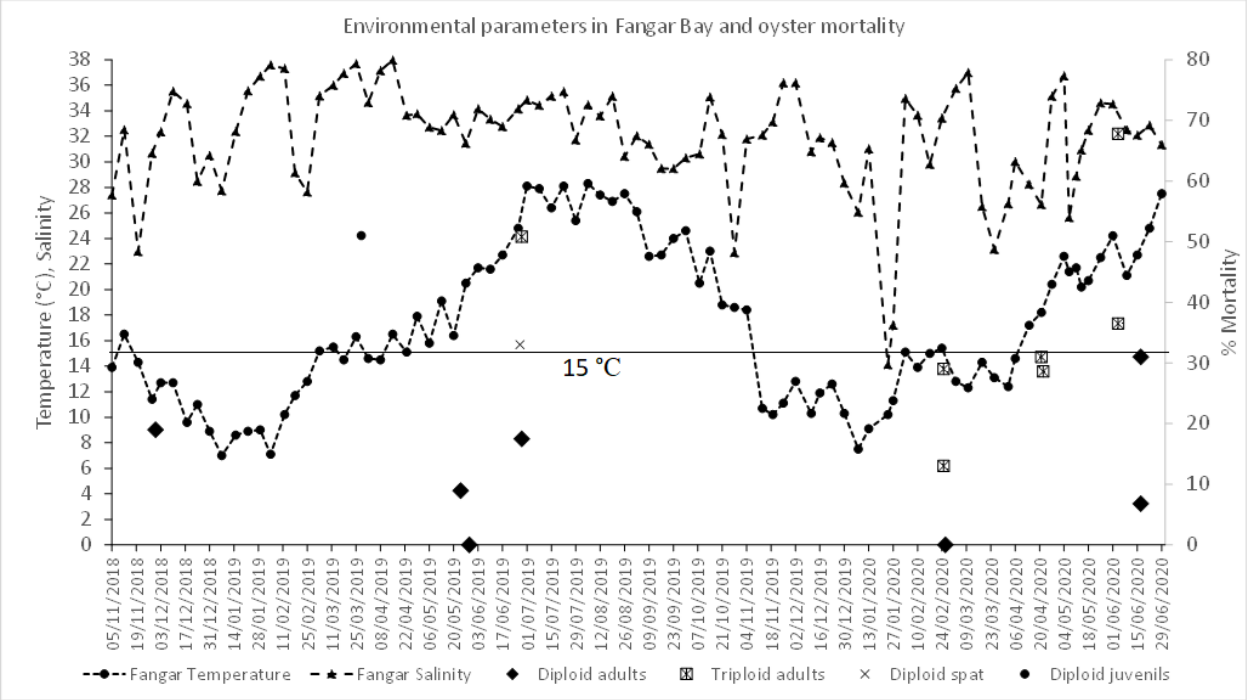


Figure 2. Environmental parameters (temperature_ −•− and salinity_ −▲−) in Fangar Bay, during the study period from November 2018 to June 2020. The dates of the sampling during mortality and non-mortality events (<7% mortality) are shown in black symbols referring to ploidy and size). The horizontal line corresponds to the 15 ºC threshold for OsHV-1. The mortality and non-mortality events (<7% mortality) associated to the 16 samples taken from this Bay are indicated with black symbols that represents the age and ploidy of the oysters involved (Supplementary table 1 specifies concrete dates, age and ploidy of the oysters studied).

Herpes virus OsHV-1 prevalence in Pacific oysters

The Ostreid herpesvirus-1 (OsHV-1) was detected by qPCR assay in 10/19 of the oyster samples collected during the study period and always during mortality episodes (Supplementary table 2). The virus was detected in springtime in all oysters age groups, correlating with mortality picks. The highest prevalence registered was in juvenile, diploid, oysters was 53.0% (2019/Mar/28-C, from Fangar Bay), associated also with a high mortality (51%). However, the highest prevalence of OsHV-1 (75.0%) during the studied period corresponded also to the highest mortality recorded (68%) in adult triploid oysters (2020/Jun/04-A from Fangar Bay), in late spring 2020. Two weeks later (2020/Jun/17-A), a high prevalence of OsHV-1 (47.0%) was observed in adult diploid oysters, but in this case the mortality was 7%, whereas in sample 2020/Jun/17-B, taken at the same time from Fangar Bay, the prevalence was lower (20.0%), but the associated mortality higher (31%). The prevalence of OsHV-1 recorded in the one sample in March 2019 from juveniles *C. gigas* during the mortality episode (17% of dead oysters) in Alfacs bay was low (3.0%), when compared with the 53.0% prevalence of the virus found in the sample taken, also from juveniles

(53.0%) in Fangar Bay during the synchronous mortality episode that generated a 51% mortality. In Fangar Bay a high OsHV-1 prevalence was recorded in all age groups, in increasing order from: adult diploid 47.0% (2020/Jun/17-A), juvenile 53.0% (2019/Mar/28-C), and adult triploids 75.0% (2020/Jun/04-A). However, the higher prevalence (53.0%) of OsHV-1 along with higher mortality (51%) was observed in juvenile oysters in spring 2019 (Figure 3). A significant ($p<0.05$) correlation between the OsHV-1 prevalence was found with the mortality episodes in Pacific oysters during the study period. Regarding environmental parameters, while oxygen concentrations were consistent and similar in both bays, there was a slightly lower temperature (15 ºC vs. 16 ºC) and lower salinity (35.5 mg/L vs. 36 mg/L) in Fangar Bay compared to Alfacs Bay. There was significant correlation ($p<0.05$) of temperature with OsHV-1 presence in adult oysters in Fangar Bay when the water temperature was 15 ºC or above. Interestingly, during 2019, OsHV-1 was detected only once in diploid adult oysters (2019/May/24) at a low prevalence (3.0 %) and not in any other sample of diploid or triploid adult oysters. Neither spat nor juvenile have been included in the analysis, since the number of samples from these size classes collected for this study was low as the mortality was reported just once during this period. When OsHV-1 was detected in the oyster tissue samples, one or more bacterial species were also detected in that sampling lot (see supplementary table 2 and figures 3-6).

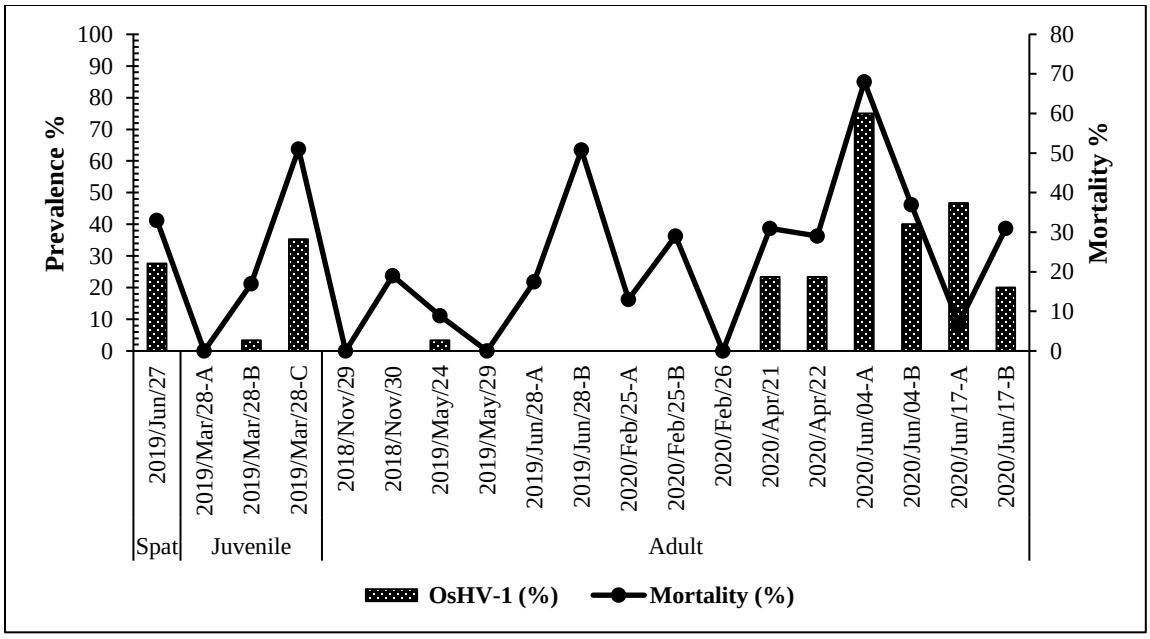


Figure 3. Prevalence of herpes virus OsHV-1 in the DNA tissue of Pacific oysters collected from Ebro Delta, Spain from November 2018 to June 2020 (bars, left Y-axis scale). Points of the line represent the % of mortality registered in the samples (right Y-axis scale). Sample 2019/Mar/28-A was taken from IRTA's experimental hatchery (OsHV1 negative in origin)

Prevalence in Pacific oysters of *Vibrio aestuarianus* and *Vibrio splendidus* clade

Vibrio aestuarianus (Figure 4) was detected by qPCR assay in oyster tissue DNA in 6/19 (31.6 %) mortality episodes (Supplementary table 2) and always in warm months i.e., April, May, and June 2019 and 2020. It was detected in oyster mortality samples from both adult and spat diploid (n = 2) and in adult triploid (n = 4). Moreover, it was also isolated five times by culture on MA plates from adult oysters, only during mortality episodes, also in the warmer months (2019/Jun/28-A & B, 2020/Apr/21, 2020/Apr/22 and 2020/Jun/04-A (Supplementary table 2). There was a significant correlation ($p<0.05$) of the *V. aestuarianus* presence with the water temperature 18 ºC and above.

The spat and juveniles being only one sample each were not included in this statistical analysis. No detection or isolation of *V. aestuarianus* was obtained from non-mortality oyster samples, thus, there was a significant ($p<0.05$) negative correlation of the bacteria isolation / detection with the mortality episodes (Supplementary table 1 & 2). The highest prevalence, by qPCR; of 30.0% (9/30), was observed in triploid adult oysters in 2019/Jun/28-B, when a cumulative mortality of 51% was recorded (Figure 4). Four (4/5) samples yield both positive qPCR detection and isolation of *V. aestuarianus*. Only in one sample (June 2019 sample A), of diploid adult oysters (mortality = 17%), there was no detection by qPCR while it was isolated by culture (being this the first isolation of *V. aestuarianus* in the Delta Ebro). Meantime, in the triploid (B) sample of the same mortality episode of June 28th, 2018, *V. aestuarianus* was both detected by qPCR and isolated on MA, however, the mortality registered in these oysters was 51%, three times higher than in the diploid lot. Furthermore, *V. aestuarianus* was not isolated from the 2019/May/24 sampling episode (mortality = 9%), although the corresponding qPCR of tissue showed a prevalence of 3.3% (1/30) which was the lowest prevalence recorded of this species throughout the study (Supplementary table 1 & 2).

A proxy to estimate the abundance of *V. aestuarianus* on the pooled tissue samples was obtained on CFU's counts on MA media, based on the colony morphology (Table 2); followed by qPCR confirmation. CFU's counts of *V. aestuarianus*-like colonies ranged between 1.5E+02 ±3.7E+01 to 3.4E+05 ±2.9E+04 cfu/ml, except for the lower mortality registered (9%), where no growth of *V. aestuarianus* was obtained, there was always grown from the pools, with a common significant ($p<0.05$) trend with mortalities (Table 2). The data also shows that during low mortality episodes i.e., up to 17% mortality, the qPCR for *V. aestuarianus* was low or negative. Nevertheless, colonies compatible with the species morphotype were isolated on MA media from the same samples i.e.,

1.5E+02 ±3.7E+01 cfu/ml. While in another low mortality sample of 2019/May/24 (9%) mortality the opposite happened, there was a low detection by qPCR (3.0%) while no growth was observed on MA media. However, in all other four samples, having mortality of 29% and above, *V aestuarianus* was both detected and isolated on MA media. Even though the morality percentage (%), the qPCR and the cfu/ml values have a common trend, this was not significant; ($p>0.05$). It is important to keep in mind that although all samples came from the same oysters, there was no standard curve to correlate the qPCR- Ct values to the CFUs counted on MA. Therefore, the qPCR assay (Saulnier et al., 2017) was being used as a +/- assay to quickly identify which oyster tissue samples were *V. aestuarianus* positive, i.e., were carriers. Whereas the CFUs were based on counts of *V. aestuarianus*-like colonies, specifically counted, apart from other non-*V. aestuarianus* compatible colony morphologies. Therefore, table 2 presents the data on the two separate *V. aestuarianus* screening tools (Table 2).

Table 2: Prevalence % of *V. aestuarianus* in the Pacific oyster tissue samples by qPCR (Saulnier et al., 2017) on individual animals. and CFU's/ml of *V. aestuarianus* -like colony counts on pooled tissues homogenates (5 animals per pool), comparting the correlative results for homologous sampling episodes. n = 30.

S. No. as of SM table 1	**Age**	**Ploidy**	**Time Period**	**Mortality (%)**	***Vibrio aestuarianus* in oyster tissue by qPCR (%)**	***V. aestuarianus* like average CFU's/ml ± S.D.**
6	Adult	Diploid	2019/May/24	9	3.3	ND
9		Diploid	2019/Jun/28-A	17	ND	1.5E+02 ± 3.7E+01
10		Triploid	2019/Jun/28-B	51	30.0	2.1E+04 ± 7.3E+03
14		Triploid	2020/Apr/21	31	13.3	6.3E+03 ± 2.0E+03
15		Triploid	2020/Apr/22	29	10.0	9.6E+03 ± 2.7E+03
16		Triploid	2020/Jun/04-A	68	15.0	3.4E+05 ± 2.9E+04

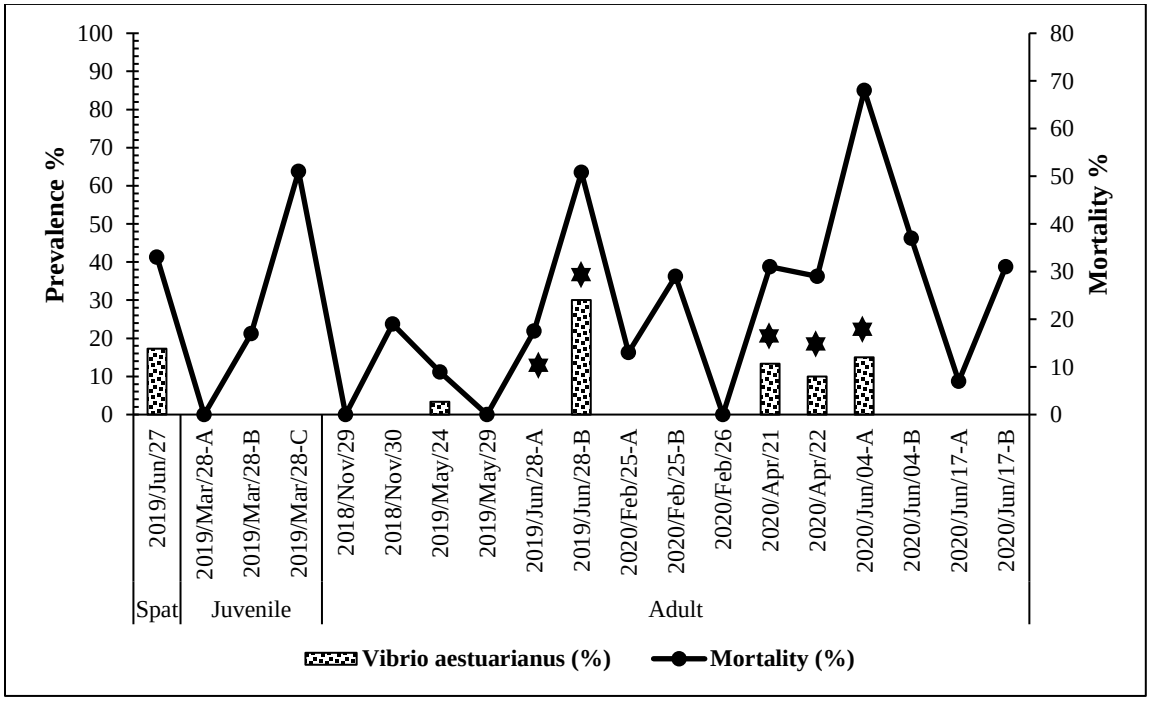


Figure 4. Prevalence of *Vibrio aestuarianus* detected by qPCR in the DNA tissue of Pacific oysters collected from Ebro Delta, Spain from November 2018 to June 2020 (bars, indicate prevalence, left Y-axis scale). Points of the line represent the percentage of mortality registered in the samples (right Y-axis scale). A ★ above the samples represent the sampling episodes where *V. aestuarianus* was isolated by culture.

V. aestuarianus and OsHV-1 were simultaneously present in spring samples from Fangar Bay both in 2019 and 2020 (Figure 5). Both OsHV-1 and *V. aestuarianus* were co-detected for the first time in 2019/May/24 adult diploid oyster samples, in a low mortality episode (9%), when the prevalence of each pathogen was also low i.e., 3.0% (1/30) each. Concurrent detection happened also in summer 2019, *V. aestuarianus* prevalence 17.0% (5/29) and OsHV-1 prevalence 28.0% (8/29), in

diploid spat from Fangar Bay (2019/Jun/27) with a 33% mortality. Whereas in 2020, co-detection happened in three adult triploid samples (2020/Apr/21, 2020/Apr/22 and 2020/Jun/04-A) coinciding with rising mortality rates (from 29% to 68%), where OsHV-1 prevalence increased concurrently from 23.0% to 75.0% during the period, while *V. aestuarianus* prevalence remained between 10.0% and 15.0%, although it was isolated in all three samples, and in the last one 2020/Jun/04-A (68% mortality), whose CFU's of *V. aestuarianus*-like colonies were the highest title of the series (3.4E+05 ±2.9E+04), whereas only three individual oyster were positive by qPCR (3/20, 15.0% prevalence). The water temperature range for all these samples varied between from 15 ºC to 25 ºC where the mortality significantly (*p<0.05)* increased when the temperature increased above 15 ºC.

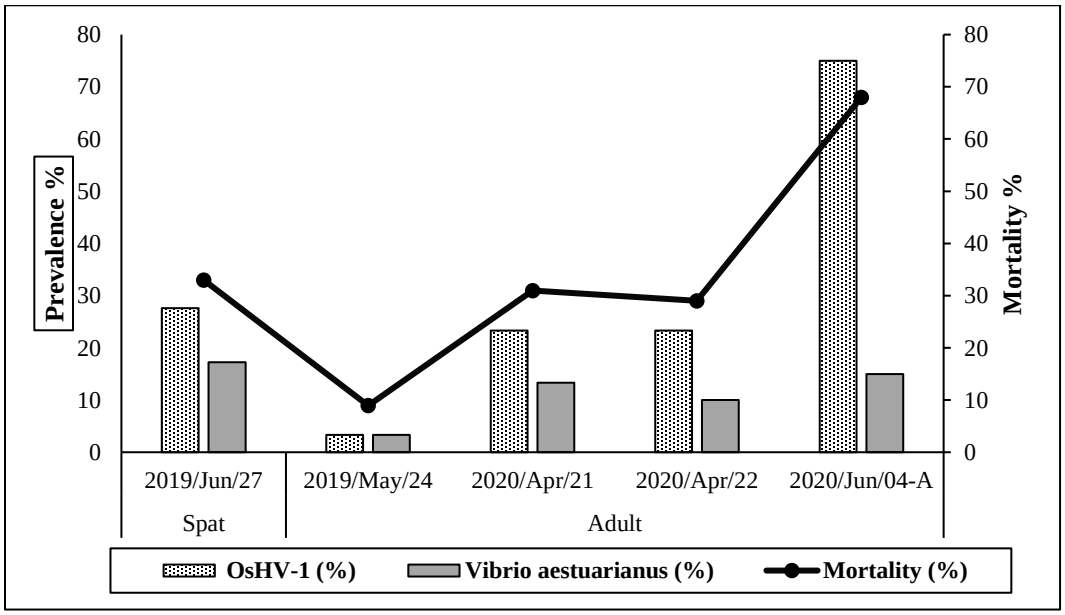


Figure 5: Co-detection and prevalence of OsHV-1 and *Vibrio aestuarianus* by qPCR in the tissue DNA of Pacific oysters collected from Ebro Delta, Spain from November 2018 to June 2020 (bars, prevalence, left Y-axis scale). Points of the line represent the percentage of mortality registered in the samples (right Y-axis scale).

Vibrio splendidus clade (Figure 6) was detected in oyster tissue DNA from 15/19 (78.9%) both during mortality and non-mortality episodes (Supplementary table 2). Isolates of *V. splendidus* like clade were obtained from all oyster samples that were positive by tissue qPCR except 2018/Nov/29 and 2018/Nov/30, (Supplementary table 1 & 2). No significant ($p>0.05$) correlation of *V. splendidus* clade by qPCR, was found with mortalities in any age group or ploidy of Pacific oysters in relation to temperature or salinity was found during the study.

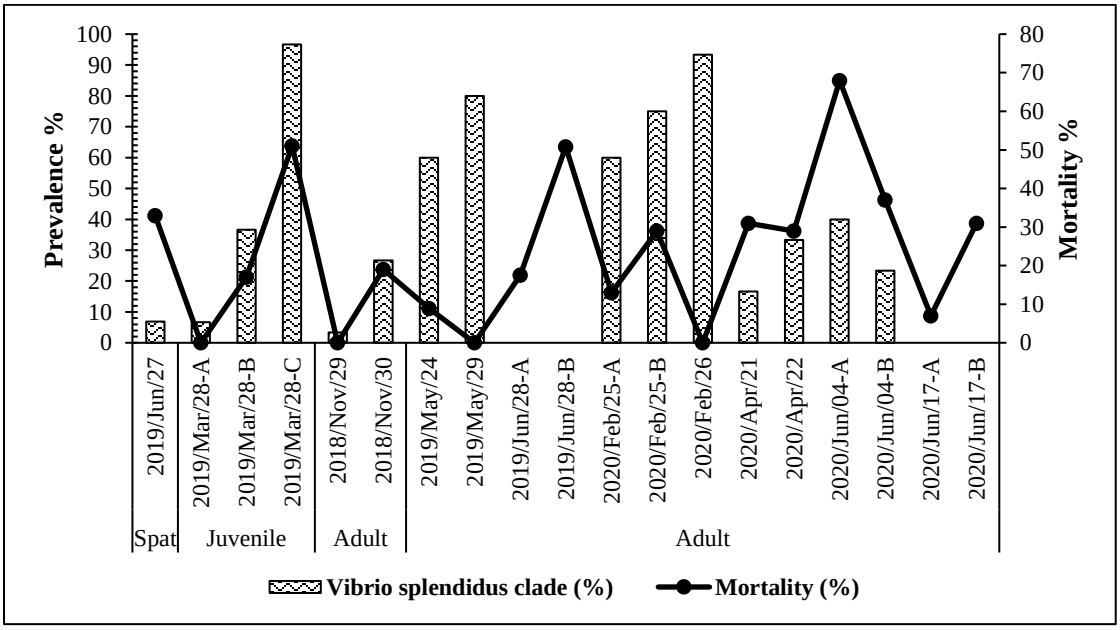


Figure 6. Prevalence of *Vibrio splendidus* clade detected by qPCR in the tissue DNA of Pacific oysters collected from Ebro Delta, Spain from November 2018 to June 2020 (bars, prevalence, left Y-axis scale). Points of the line represent the percentage of mortality registered in the samples (right Y-axis scale). No significant ($p>0.05$) correlation of *V. splendidus* clade was found with the mortality episodes in Pacific oysters.

Prevalence in Pacific oysters of *Arcobacter*-like spp.

Arcobacter-like spp. was detected in high prevalence (up to 90.0%) in all, but one sample of tissue DNA of all age groups of Pacific oysters, from both mortality and non-mortality episodes (Figure 7 & SM table 2). Except for one non mortality sampling episode, 2018/Nov/29. The highest prevalence of *Arcobacter*-like spp. (90%) was found in winter, from a non-mortality episode (2020/Feb/26). In mortality episodes samples, high prevalence (80%) was detected both in winter (2020/Feb/25-B) with a mortality of 29%, and in summer (2020/Jun/04-A), with higher mortality (68%), (Figure 7). After enrichment, *Arcobacter*-like spp. colonies were obtained from all samples which were positive by the Arcobacter 23S qPCR assay, except 2018/Nov/30 mortality (19%) sample having only a 6.7% prevalence by qPCR analysis of tissue DNA (Supplementary table 2). Thus, there was no significant ($p<0.05$) correlation of the general *Arcobacter*-like spp. with the mortality episodes, age group/ploidy of the oysters, temperature, and/or salinity of the bay. Further characterization of the *Arcobacter*-like spp. by sequencing of *rpoB* gene revealed that *M. marinus*, a specie of *Arcobacter*-like spp., was significantly ($p<0.05$) found only during the mortality episodes as explained below.

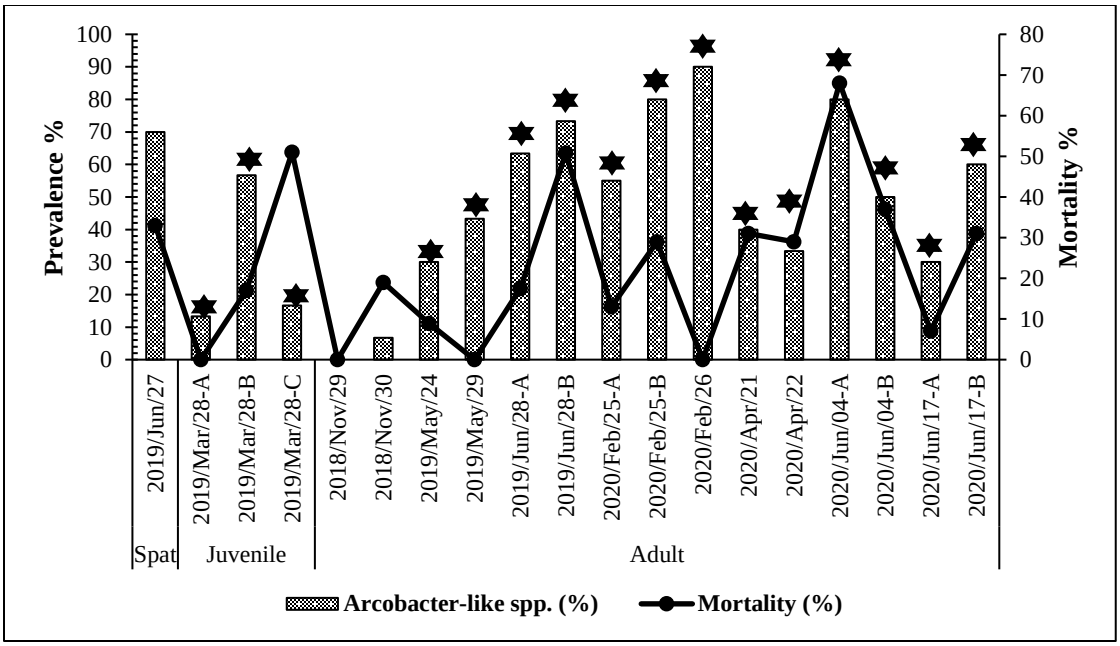


Figure 7. Prevalence of *Arcobacter*-like species by qPCR in tissue DNA tissue of Pacific oysters collected from Ebro Delta, Spain from November 2018 to June 2020 (bars). No significant (*p>0.05*) correlation of *Arcobacter*-like spp. was found with the mortality episodes in Pacific oysters. A ★ above the samples represent the sampling episodes where *Arcobacter*-like spp. were isolated by culture.

Genotyping and molecular characterization of *Arcobacter*-like spp.

The isolates of *Arcobacter* like spp. collected from *C. gigas* tissue homogenates during the study period, in both mortality and non-mortality episodes showed varying ERIC-PCR banding patterns, indicating the existence of several different genotypes of *Arcobacter*-like spp. No specific ERIC-PCR-based genotype was found associated particularly with mortality samples, rather, different genotypes were isolated from all samples. A total of 37 ERIC-PCR genotypes were identified from 141 *Arcobacter*-like isolates recovered from both mortality and non-mortality samples. Of all these genotypes, 73% (27/37), corresponded to 80 % (113/141) of the isolates that were identified as *M.*

marinus, while 27% (10/37) genotypes, corresponding to 20% (28/141) of the isolates, which were genotypes of unknown *Arcobacter*-like spp., based on the sequencing and phylogenetic analysis of the *rpoB* gene.

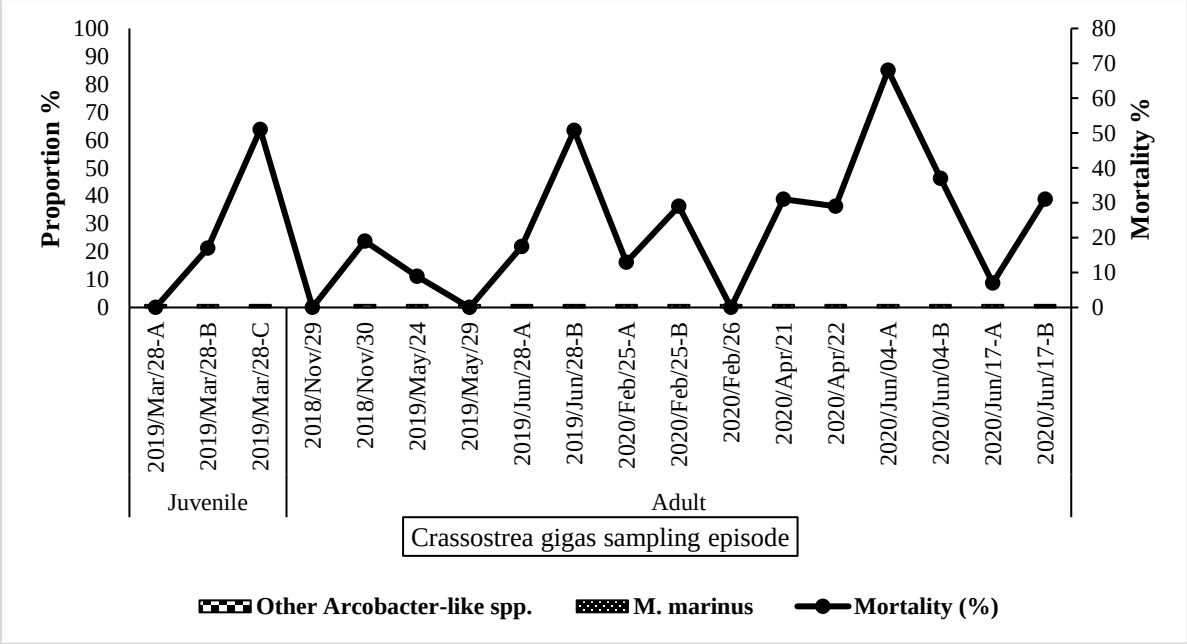


Figure 8: Proportion (left Y-axis) of *Malaciobacter marinus* and other *Arcobacter*-like species (calculated by number of isolates of each species /total number of isolates x 100) from different mortality and non-mortality samples of Pacific oysters (right Y-axis). There was a significant ($p<0.05$) correlation of *M. marinus* with mortality in juvenile and adult diploid/ triploid Pacific oysters collected from Fangar Bay during the study.

The phylogenetic neighbor-joining tree (Figure 9) of 425 bp of *rpoB* shows that most of the isolate's group together with the type strains of *M. marinus* MDC1741^T having a high bootstrap value of 99%. The NCBI nucleotide blast analysis search results of the *M. marinus* strains showed a 99.6% to 100% similarity with *M. marinus* MDC1741^T strain (Accession No. JF803185.1). This

shows that these isolates belong to the *M. marinus* species. The isolates IRTA-19-132 and IRTA-19-136 showed a 97.7% and 98.6% similarity, respectively, with *Malaciobacter neptunis* F146-38^{T} (Accession No. LT904787.1). The isolates IRTA-19-133 and IRTA-19-143 showed a 96.5% and 96.0% similarity with *Halarcobacter salis* F155-33^{T} (Accession No. LT904791.1). The isolated IRTA-20-195 showed an 89.2% similarity with *Arcobacter* sp. MDC1765 (Accession No. JF803194.1). The closest similar of isolate IRTA-20-148 was *Poseidonibacter parvus* LPB0137^{T} (Accession No. CP019070.1), showing an 89.4% similarity. The closest similar of isolates IRTA-19-147, IRTA-19-158 and IRTA-20-150 was *Poseidonibacter lekithochrous* DSM 100870^{T} (Accession No. CP054052.1), showing 87.4%, 87.1% and 86.2%, respectively. The isolates IRTA-19-149 had an 85.8% similarity with *Arcobacter* sp. F155-44 (LT904793.1) while IRTA-19-236 had a 96.2% similarity with *Arcobacter* sp. ARW1-2F2 (Accession No. CP100595.1). There was a significant ($p<0.05$) correlation of *M. marinus* with mortality in juvenile and adult diploid/ triploid Pacific oysters collected form Fangar Bay during the study.

RESULTS AND DISCUSSION

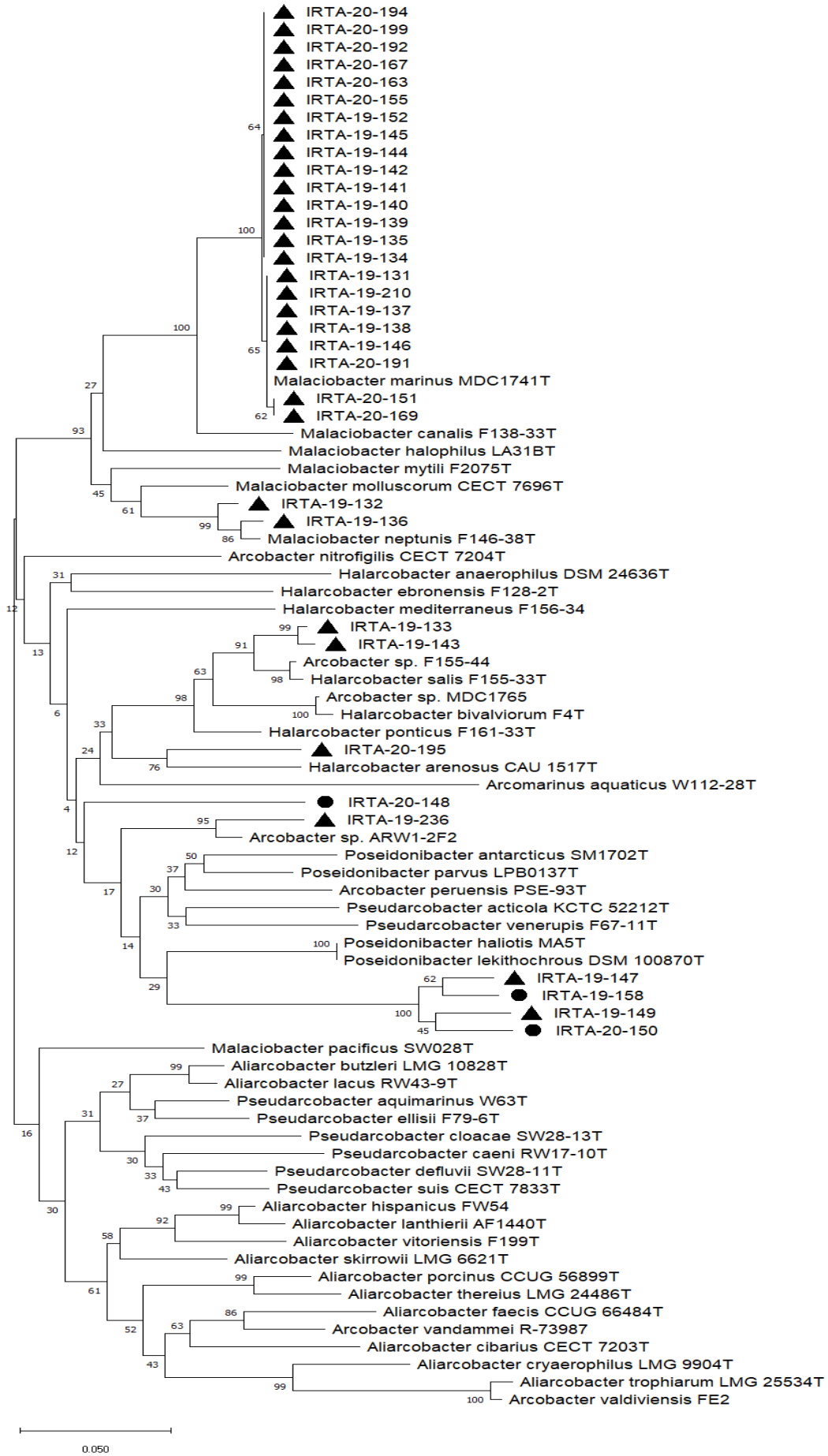


Figure 9. Phylogenetic analysis, using Neighbor-Joining (NJ) tree algorithm with 1000 bootstrap replicates, of 425 bp long *rpoB* gene sequences form *Arcobacter*-like isolates recovered from mortality and non-mortality episode samples collected from November 2018 to June 2020 in the Ebro Delta, Spain. The solid black triangle, ▲ (n = 31) shows the *Arcobacter*-like isolates from mortality episode samples while the solid black circle, ● (n = 3) represents non-mortality episode isolates.

Polymicrobial recovery on mortality episodes

In some episodes of mortality, co-occurrence of viral and/ or bacterial agents happened (Table 3). However, in some cases, the detection of *V. aestuarianus* was only culture-based, since the qPCR gave negative results, such was the case of sample 2019/Jun/28A (mortality = 17%), However the synchronic triploid adult 2019/Jun/28-B (mortality = 51%) was found carrying both *M. marinus* and *V. aestuarianus* by qPCR test (table 3), and also recovering both from the homogenates of oyster pools (Supplementary table 1).

Moreover, the adult oyster 2020/Jun/04-B, 2020/Jun/17-A (mortality = 7%) and 2020/Jun/17-B (mortality = 31%) samples carried both OsHV-1 and bacterial *M. marinus*, and no *V. aestuarianus* (Table 3). Similarly, the diploid juvenile oyster 2019/Mar/28-B (mortality = 17%) and 2019/Mar/28-C (mortality = 51%) were also coinfected by OsHV-1 and *M. marinus* (Table 3), there is no date on *V. aestuarianus* culture for those samples, but the bacteria was not detected by qPCR. While diploid adult 2019/May/24 (mortality = 9%), triploid adult 2020/Apr/21 (mortality = 31%), 2020/Apr/22 (mortality = 29%) and 2020/Jun/04-A (mortality = 68%) had an OsHV-1 viral coinfection along with poly-bacterial *V. aestuarianus* and *M. marinus* infection (Table 3).

Table 3. Polymicrobial detection of OsHV-1, *Vibrio aestuarianus* and *Malaciobacter marinus* infection in Pacific oyster *Crassostrea gigas* tissue samples by qPCR assays from Ebro Delta, Spain. n= 30; the prevalence is shown in terms of % for each pathogen.

S. No. as of SM table 2	**Age**	**Time Period**	**Mortality (%)**	***OsHV*-1 (%)**	***Vibrio aestuarianus*- (%)**	***Malaciobacter marinus* (%)**
8	Spat	2019/Jun/27	33	27.6	17.2	ND
4	Juvenile	2019/Mar/28-B	17	3.3	ND	56.7
5		2019/Mar/28-C	51	53.3	ND	16.7
6	Adult	2019/May/24	9	3.3	3.0	30.0
10		2019/Jun/28-B	51	ND	30.0	64.5
14		2020/Apr/21	31	23.3	13.3	31.6
15		2020/Apr/22	29	23.3	10.0	31.0
16		2020/Jun/04-A	68	75.0	15.0	55.2
17		2020/Jun/04-B	37	40.0	ND	44.5
18		2020/Jun/17-A	7	46.7	ND	30.0
19		2020/Jun/17-B	31	20.0	ND	40.2

ND= Not detected

Development of a PCR and qPCR assay for the fast recognition of *M. marinus* (Mm-rpoB)

The designed Mma-rpoB primers efficiently amplified the 135 bp long fragment of *M. marinus* DNA (Figure 10). The optimal annealing temperature for these primers, determined using a gradient program that included several different annealing temperatures, was 66.3 ºC. From the sequence alignment we know that most strains have no site for primer annealing. *M. canalis* though was very similar and for this reason the conditions to remove non-specific primer binding needed to be carefully adjusted and tested. Many of the other species were eliminated by "in silico" testing. This was also confirmed by the absence of amplification from 15 other *Arcobacter*-like species (Table 1) mostly of marine origin, used as controls in the assay for specificity.

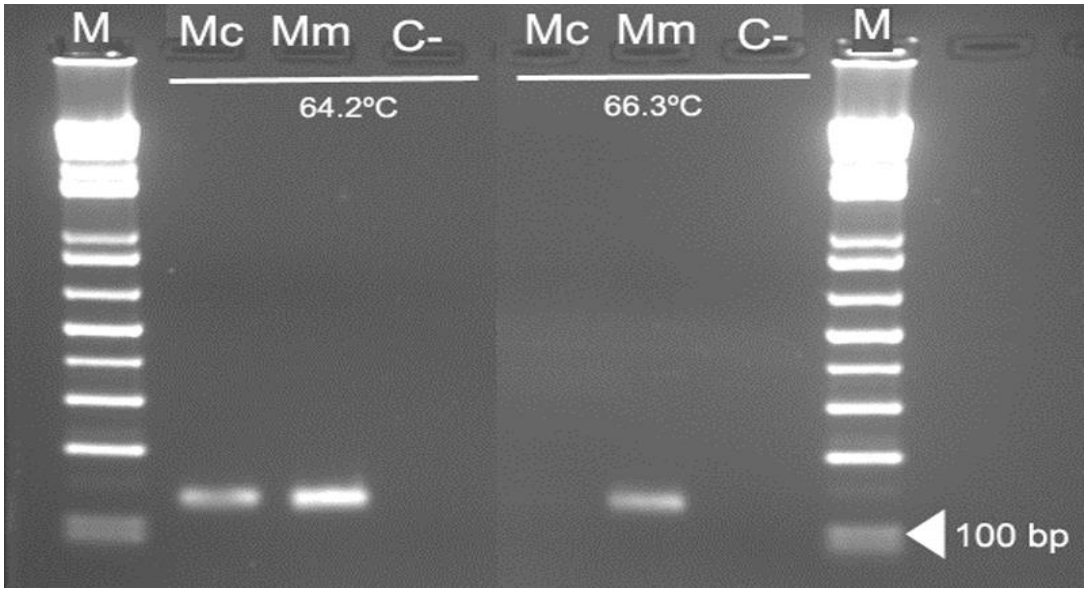


Figure 10: Optimization of Mm-rpoB primers for amplification of 135 bp DNA fragment of the *rpoB* gene of *Malaciobacter marinus*. Using an annealing temperature of 66.3 ºC specificity for *M. marinus* DNA (IRTA-19-132). Lanes: M= Molecular Marker; Mc= *Malaciobacter canalis*; Mm= *Malaciobacter marinus*; C- = Control Negative.

Using the qPCR assay, the specific amplification from *M. marinus* strains was observed at 62.5 ºC. The positive amplification occurred at different dilutions ranging from 5ng/µL to 50 fg/µL of pure *M. marinus* gDNA showing Ct values from 19 to 37. There was no amplification from dilutions below 50 fg/µL for pure *M. marinus* gDNA. Positive amplification was observed in samples of oyster tissue DNA (50 ng/µL) spiked with dilutions of *M. marinus* (IRTA-19-132) gDNA in concentrations from 5ng/µL to 0.5 pg/µL resulting in Ct values from 19-35. As compared with the pure *M. marinus* genomic DNA dilutions, the addition of 50 ng of oyster gDNA resulted in the loss of amplification from 50 fg/µL to 0.5 pg/µL (loss of 1 log). Amplification was also

observed from the gDNA extracted from 10^8, 10^7, 10^6, 10^5, and 10^4 CFU's/mL from *M. marinus* bacterial suspensions (Data not shown), while there was no amplification from the lower dilutions. The calculation from the standard curve showed a qPCR efficiency (*E%*) of 90%. The LoD was 50fg/µL for pure *M. marinus* genomic DNA, while in the case of oyster DNA spiked with *M. marinus* genomic DNA, the lowest dilution detected was 0.5 pg/µL.

Presence of virulence genes in *Arcobacter*-like isolates

Nine putative virulence genes i.e., *ciaB, cj1349, hecA, irgA, mviN, cadF, hecB, pldA, and tlyA* were studied in these 37 representative genotypes of *M. marinus* (n = 10) and other *Arcobacter*-like (other than *M. marinus*) isolates (n = 27). Only 3 out of 9 virulence genes i.e., *ciaB, cj1349,* and *tlyA* were detected in all representative genotypes (Figure 11). The presence of *ciaB* gene was present in 11.1% (3/27) and 30% (3/10) in *M. marinus* and other *Arcobacter*-like spp., respectively. The presence of *cj1349* gene was 3.7% (1/27) and 10% (1/10) in *M. marinus* and other *Arcobacter*-like spp., respectively. While the *tlyA* gene presence was 11.1% (3/27) and 10% (1/10) in *M. marinus* and other *Arcobacter*-like spp., respectively (Figure 11). The presence of these virulent genes in *Arcobacter*-like spp. indicate that these species could have the capacity to invade the host cells.

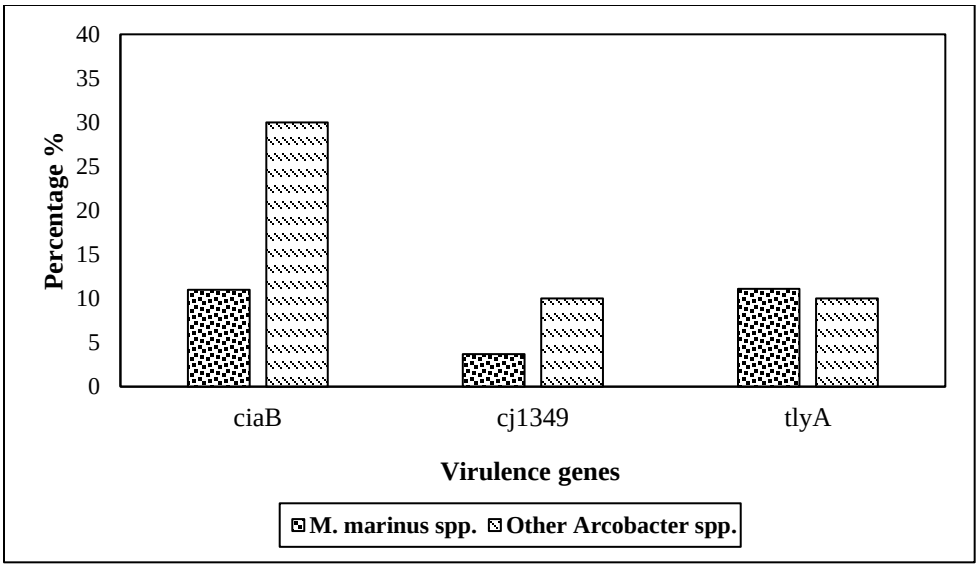


Figure 11. Prevalence of virulent genes in *Malaciobacter marinus* and other *Arcobacter*-like spp. isolated from Pacific oyster samples collected from Ebro Delta, Spain.

Discussion

In the Ebro Delta harvesting area of *C. gigas*, an increase in mortality (80%) was observed in spat in 2007 and has become an annual occurrence since then, when the water temperature reaches 16 ºC in autumn and/or spring (Roque et al., 2012; Carrasco et al., 2017). Due to these events, the production of *C. gigas* has declined and it is highly variable depending on the intensity of the mortality episodes, largely attributed to viral (herpes virus OsHV1_microvar) or bacterial pathogens (*Vibrio* spp.) (Fuhrmann et al., 2019). The mortalities in oyster spat and juveniles have been mostly associated with OsHV-1 (Friedman et al., 2005; Segarra et al., 2010 & 2014; Keeling et al., 2014; Carrasco et al., 2017; Lorgeril et al., 2018; Degremont et al., 2021; Jiang et al., 2021) while *Vibrio* spp., and specifically *V. aestuarianus* causes mortality in adult oysters (Lacoste et al., 2001; Gay et al., 2004; Garnier et al., 2007; Azema et al., 2017; Degremont et al., 2021; Jiang

et al., 2021). The present study aimed to address the losses of commercial size *C. gigas*, considering that mortalities of unknown etiology in this age group and juveniles that are becoming recurrent in the Ebro Delta area and had been previously linked to *V. aestuarianus*, through its detection by qPCR (Carrasco et al., 2018; Lasa et al., 2019). Opportunely, the species was isolated for the first time, to our knowledge, in the present study in June 2019 during a mortality episode affecting adult *C. gigas* where the water temperature was at 24 ºC and rising (Vezzulli et al., 2010). The detection of OsHV-1, *V. aestuarianus, V. splendidus* clade and *Arcobacter*-like spp. represent a mixed infection in the spat oysters collected on 2019/June/27; likewise, this had been previously reported in France that a mix infection of OsHV-1 and *V. aestuarianus* was found responsible for mortality in *C. gigas* (Azema et al., 2017).

In this study, *Arcobacter* sp. was both detected by PCR and isolated ubiquitously from *C. gigas* tissue, both in mortality and in non-mortality episodes from oysters of all age classes; therefore, *Arcobacter spp*., presence could not be correlated with *V. aestuarianus* detection/ isolation in these mortality events. Conversely, *M. marinus* detection and isolation was significantly associated with *C. gigas* mortality episodes in the Fangar Bay of the Ebro Delta, which opens future research questions regarding its role in pathogenicity and polymicrobial infections. Previous studies have reported that both OsHV-1 and *V. aestuarianus* causes the mortality in all age groups of pacific oysters together in a mix infection (Azema et al., 2016; Degremont et al., 2021), while *V. aestuarianus* alone can cause mortality in adult commercial size oysters (Green et al., 2016; Lupo et al., 2019; 2020; Degremont et al., 2020; Mesnil et al., 2022). In this work, we detected both OsHV-1 and *V. aestuarianus* in adults and spat affected by mortality, but not consistently; the concomitant factors affecting the potential synergies between both pathogens needs further investigations in our area. As previously demonstrated by Petton et al. (2015), before the juvenile

oyster mortality, a colonization of *Vibrio* and then followed by the OsHV-1 replication can occur, which debilitates the animal's health and leads to the mortality of the oyster. Moreover, they also demonstrated that in the absence of vibrios, even a large viral load of OsHV-1 was insufficient to cause harm to the oyster. Nonetheless, the synergy of OsHV-1 and *V. aestuarianus* had far more severe implications on oyster mortality. Interestingly, although the present study only includes three samples of juvenile oysters (2019/Mar/28-A, B and C), they were all collected in March 2019, during mortality episodes that occurred in both bays (water temperature 15-16ºC). The juveniles from IRTA's hatchery (produced in an OsHV-1 free environment) did not register any mortality, those from Alfacs Bay registered a low mortality (17%), whereas oysters from Fangar Bay died at a 51% rate. The prevalence for detection of OsHV1 among IRTA oysters and those from Alfacs and Fangar bays followed a similar trend: 0.0, 3.3 and 53.3%, respectively. Whereas *V. aestuarianus* was not detected in any of them. In contrast, both *V. splendidus* and *Arcobacter*-like spp. were detected in all three samples of juvenile oysters, and in the case of *V. splendidus*, the prevalence recorded by qPCR increased with the mortality; in this case the IRTA sample carried a low prevalence (6.7%), Alfacs oyster 36.7% and juveniles from Fangar 96.7%, the highest prevalence recorded in the whole series. Interestingly, species of the *V. splendidus* clade have been reported to be pathogenic to juvenile oysters (Bruto et al., 2017), in this work, *V. splendidus* clade representatives were present across the studied samples both when mortalities occurred and in the absence of mortalities, thus, a pathogenic role could neither be attributed, nor discharged for this bacterium, the only trend found was in the above described from three juvenile samples. The lack of specificity of the diagnostic test used/ available for *V. splendidus* clade make it difficult to efficiently investigate the role of these bacterial clade in oyster mortality. The lack of specificity in diagnosis using the method of Saulnier et al (2017) has been reported previously

by Nasfi et al. (2015), where he had isolated members of the *V. splendidus* clade from *C. gigas* with both mortality and non-mortality samples and could not attribute a specific strain to these mortalities. Interestingly, still looking at the juvenile mortality episode, although *Arcobacter*-like species were detected by PCR in the three samples, *M. marinus*, was isolated only in *C. gigas* presenting mortality, though not in IRTA's oyster stocks where no mortality was registered. In this study the specific presence of *M. marinus* in *C. gigas* juveniles experiencing mortalities, together with other potential pathogens such OsHV-1 has been found to be in accordance with previously reported presence of *Arcobacter* spp. in juvenile Pacific oysters during mortality events (Lasa et al., 2019; Petton et al., 2021; Richard et al., 2021), Moreover, in our work *M. marinus* has been seen as more likely to be a concomitant agent with the mortality event.

In adult oysters, OsHV-1 was detected in samples collected during five sampling occasions over the three years when the water temperature was in the range of 17 to 23 ºC in Fangar Bay, which is slightly higher than the usual temperature in the study area during outbreaks of OsHV-1 affecting spat (Carrasco et al., 2017). Fleury and coworkers (2020) found that the rise in temperature was associated with OsHV-1 causing mortalities in *C. gigas*. While the studies by Pernet et al. (2012) and Renault et al. (2014) have also reported that OsHV-1 outbreaks in *C. gigas* occur when the water temperature is between 16 ºC to 24 ºC, the same range recorded in the present work.

Although initially OsHV-1 was considered a pathogen of spat and juvenile Pacific oysters, it has been demonstrated that it can also cause mortalities in adult oysters. (Segarra et al., 2014; Degremont, 2013; Hick et al., 2018). In agreement with this work, in a preceding study on *C. gigas* mortalities in the Delta Ebro producing area, (2016-218), there was also detection of both OsHV1-microvariant and *V. aestuarianus* in adult oyster (VIVALDI, EU project (WPI, D1.6, 2020). Co-infection cases of OsHV-1 and *Vibrio* spp., specifically *V. aestuarianus*, in oysters have been

previously reported elsewhere (Pernet et al., 2012; Petton et al., 2013; Solomieu et al., 2015; Azema et al., 2016; Green et al., 2016; Alfaro et al., 2019; Mandas et al., 2020). Although coinfections with OsHV-1 and *V. aestuarianus* have also been revealed in mortality episodes in this study, it is worth noticing that adult diploid oysters affected by mortality, in June 2020, were harboring OsHV-1 and *M. marinus*, but no vibrios were detected.

Vibrio spp. are prominent bacteria in the marine environment, associated with flora, fauna, and in a free-living state (Kaneko & Colwell, 1973, McCarter, 1999; Grau et al., 2005; Pruzzo et al., 2005a; Vezzulli et al., 2009; Wendling et al., 2014). Many roles have been attributed to representatives of the genus *Vibrio*, from associates of marine fauna, as commensals (Restrepo et al., 2018), probiotic use (Desriac et al., 2010), to pathogenic (Kalatzis et al., 2018). In the present study, *V. aestuarianus* was detected in adult oysters with a significant correlation ($p < 0.05$) of *V. aestuarianus* in triploid oyster at a temperature above 18 ºC, which could indicate that triploid oysters may be more susceptible to mortality caused by this bacterium than diploid oysters during summer season. A similar finding has been reported by Houssin et al. (2019) where adult triploid oysters had significantly higher mortalities than diploid counterparts in Normandy, France. These results agree with those previously reported in experimental challenge studies at 18 ºC and 20 ºC (De Decker et al., 2011) and 21 ºC (Azema et al., 2016). *V. aestuarianus* was first detected in the Ebro Delta in 2016 by molecular methods (VIVALDI Project WP1, deliverable 1.6; Garnier et al., 2007; Carrasco et al., 2018; Lasa et al., 2019; Lupo et al., 2019) demonstrating it to be associated to mortalities in adult oysters, but it was not isolated on culture media from the oyster tissues at that time. Having *V. aestuarianus* isolates from mortality episode of *C. gigas* will allow to conduct paramount pathogenicity studies (Azema et al., 2016; 2017).

The *V. splendidus* clade has been found associated with mortality outbreaks in several aquaculture species, including *C. gigas* (Saulnier et al., 2010; Nasfi et al., 2015; Green et al., 2019). Specifically, *V. splendidus, V. tubiashii*, and *V. anguillarum* are the most common *V. splendidus* clade species pathogenic to *C. gigas* (Wendling et al., 2014). In the present study, *V. splendidus* clade were detected with OsHV-1 and isolated from 78.9% (15/19) of the samples, some with a prevalence as high as 96.7% from a spring mortality episode involving *C. gigas* juvenile (2019/Mar/28-C, mortality = 51%). Although the hypothesis of a potential positive synergy among the virulence factors of these two pathogens resulting in an increased mortality should be investigated further (Alfaro et al., 2019; Pernet et al., 2012), the results from the present work would not support such a postulate. Thus, high prevalence was recorded in adult oyster for *V. splendidus* clade representatives both during the winter 2020 mortality episode (29% mortality), but also in several samples not linked with mortality, including the highest *V. splendidus* clade prevalence of 93.3% in the samples 2020/Feb/26. The absence of a significant ($p<0.05$) correlation of this bacterial clade to mortalities in the studied zone and period, does not support it as an etiological agent in *C. gigas* mortalities, considering the current lack of specificity of the identification method (Vezzulli et al., 2018; Destoumieux-Garzón et al., 2020). Although this clade is considered part of a healthy oyster's microbiome, there may be some species/strains that can assist in causing mortality when accompanied by other pathogens or by temperature stimulation (Gay et al., 2004; Wendling et al., 2014; 2015; Solomieu et al., 2015; Green et al., 2019). Furthermore, the diagnostic qPCR detection assay used (Saulnier et al., 2017) is not species-specific for *V. splendidus*, it can detect at least 16 different bacterial species grouped together, which compromises the conclusions that can be drawn for its use in epidemiological studies. Bearing this in mind, our results agree with those of a previous study in the Ebro Delta, where

members of the *V. splendidus* clade were also detected in both non-mortality and mortality samples (Carrasco et al., 2018). Previous studies of the *V. splendidus* clade have found it associated with summer mortalities, mostly in juvenile oysters (Lacoste et al., 2001), although there was no significant correlation ($p<0.05$) found between water temperature and *V. splendidus* clade prevalence in adult Pacific oysters during the present study. It has also been reported that the *V. splendidus* clade related strains are abundant in *C. gigas* and coastal waters when the water temperature is below 20 ºC, while other core *Vibrio* spp. increase when the temperature rises above 20 ºC (Wandling et al., 2014). As the *V. splendidus* clade, as previously mentioned, consists of 17 different species (Yardimci, 2020), in the future it will be important to dedicate work to recover isolates to identify them to species level and study their role in shellfish mortalities. Moreover, some *V. splendidus* clade species, though normally non-pathogenic to oysters, can be pathogenic under certain conditions i.e., temperature fluctuation, presence of other bacteria in the microbiota cohort, acquisition of virulence factors or growth threshold (Nasfi et al., 2015), therefore, cross examination with key environmental drivers and microbiota communities is also very advisable.

Arcobacter-like spp. are ubiquitous in nature (Salas-Masso et al., 2018; Rahman et al., 2020) and several species have been isolated from marine environments and shellfish in the Ebro Delta (Figueras et al., 2011; Levican et al., 2012; 2014; 2015; Collado et al., 2014; Salas-Masso et al., 2016; 2018). In the present study, *Arcobacter*-like spp. have been detected in oyster tissue in 94.7% (18/19) samples in both mortality and non-mortality samples (Figure 6). The highest prevalence for *Arcobacter*-like, 90% was found in non-mortality episode sample (2020/Feb/26) during the colder months. *Arcobacter* and the *V. splendidus* clade both contain several species that are potentially of interest regarding pathogenicity. Although, *Arcobacter*-like spp. have been reported from many locations (Levican et al., 2012; 2014; Laishram et al., 2016; Salas-Masso et al., 2016;

Leoni et al., 2017; 2018; Zhang et al., 2019; Mudadu et al., 2021; Richard et al., 2021) and specifically during mortality events in the microbiome and hemolymph of oysters (Lokmer & Mathias Wegner 2015; King et al., 2019; Lasa et al., 2019), no cultures were isolated in those mortality studies to identify and functionally test the species. During the present study we isolated in culture the more abundant species from mortality episodes in Pacific oysters. *Arcobacter*-like spp. in general (excluding *M. marinus* sp.) was not associated with mortality ($P>0.05$). Although it was detected in the adult mortality episode samples, with high prevalence of 80% both in winter, Feb-2020 (mortality = 29%), and in late spring, Jun-2020 samples (mortality = 68%), however it was equally present in non-mortality samples. This could be explained because some species are found commonly in the marine environment and could be part of the normal microbiota of a filter feeder like *C. gigas*, including *Malaciobacter marinus* (syn. *Arcobacter marinus*), that will be accumulated by the shellfish (Lasa et al., 2019; King et al., 2019). The molecular characterization and comparative analysis of the mortality and non-mortality *Arcobacter*-like isolates revealed that *Malaciobacter marinus* is abundantly present in mortality episode samples (Figure 8). In fact, this specie was recovered from mortality samples and shown to have significant correlation ($p<0.05$) to mortalities; moreover, it was not found in contrasted samples from non-mortality events. The molecular characterization and phylogenetic analysis of the *rpoB* gene DNA sequences revealed that about 80.1% of the *Arcobacter*-like spp. isolated from the mortality episodes were *M. marinus* sp. (99.6%-100% similarity). Consequently, this species was suspected to have a possible role in the etiology of mortality in Pacific oysters. Therefore, it was decided to design a species-specific primer set for amplifying the *rpoB* gene of *M. marinus*, to be able to have a fast-screening tool to assess the real abundance of this species in the mortality episodes as well as in non-mortality samples. Considering that *M. marinus* could suppose a risk in the production of *C. gigas*, a qPCR

assay for the surveillance has been developed, as a tool to assist in epidemiology and screening studies of *M. marinus* in the marine environment and shellfish such as *C. gigas*. The 1 log decrease (from 50 fg/µL to 0.5 pg/µL) in detection of *M. marinus* gDNA in presence of oyster DNA should be considered when screening oyster tissue DNA to detect *M. marinus*. Although, Hausdorf et al. (2013) didn't observe such interference from spinach DNA in his qPCR assays for detection of *Arcobacter*-like spp., other studies show that this kind of interference can happen (Onderdonk et al., 2008). The decrease in detection (not detectable below 10^4 CFU's/mL) from the 10-fold serial dilutions of *M. marinus* cell suspensions could be due to the reason that efficiency of DNA recovery from different matrices is around 30% or lower (Kralik & Ricchi, 2017), while DNA already extracted can be diluted to near an extinction point giving a lower limit of detection. These molecular based tools will help in surveillance for this *Arcobacter*-like species and to detect it efficiently and rapidly to aid in facilitating a proper management response to such mortality events.

This marine *Arcobacter*-like sp. was first isolated from seawater in South Korea in 2010 (Kim et al., 2010) and later isolated in the current study area (i.e., Ebro Delta, Spain) from oysters and other shellfish spp. in 2016 (Salas-Masso et al., 2016). To our knowledge, it has not been isolated in any other area or location and has not yet been studied for its role in shellfish mortalities, specifically *C. gigas*. The presence of only three of the nine virulence genes of *Arcobacter*-like spp. (Douidah et al., 2012) analyzed in the current study, indicated that these virulence factors are not abundantly present in these bacteria. Although, a low abundance of *ciaB*, *cj1349* and *tlyA* virulence genes was present, these genes are responsible for invasion, fibronectin binding and hemolysin production, respectively. In the previous study of Levican and coworkers (2013), where five virulence genes (*ciaB, cadF, cj1349, hecA* and *IrgA*) were studied, they found only *ciaB* gene (encoding invasion protein) in multiple *Arcobacter* isolates while the prevalence of the other four

genes were low and present only in few isolates. Several studies have reported that *Arcobacter*-like spp. could be opportunistic pathogens for *C. gigas* as their abundance increases during mortality events in the hemolymph and microbiome of *C. gigas* (Lokmer & Wegner, 2015; Green et al., 2019; King et al., 2019; Lasa et al., 2019). Yet detailed knowledge about its potential role in *C. gigas* mortalities is unknown. In the present study, for the first time a specific *Arcobacter*-like spp. i.e., *M. marinus* has been reported to be found abundantly and isolated during mortality episodes of *C. gigas*. In this study, it was also found that some additional pathogens (viral and/or bacterial) were present, including *Arcobacter* like spp. (in accordance with previous work), during mortality episodes in *C. gigas*. It has been observed indirectly that intra-host collaborations may be occurring during pathogenesis, in the OsHV-1 immune compromised oyster, which then facilitates the colonization of bacteria within *C. gigas* (Lorgeril et al., 2018).

This study concludes that temperature and microbial infection played major roles in the mortality of *C. gigas* in the Ebro Delta harvesting area, during the studied period (November 2018 to June 2020). Several known viral and bacterial pathogens including OsHV-1, *V. aestuarianus,* and *V. splendidus* clade as well as bacteria with unknown roles like *Arcobacter*-like spp. have been detected and/or isolated from the described mortality episodes in the Ebro Delta, though not always associated with mortality. *V. aestuarianus* was isolated for the first time on cultural media from the Ebro Delta. Other bacterial species like *M. marinus,* with an unknown role in mortality of *C. gigas,* were abundantly detected and isolated from mortality samples. The role of this bacterial colonizer should be evaluated in shellfish, specifically in *C. gigas* by challenging experiments. This study provides a survey of selected viral and bacterial pathogens from *C. gigas* in the Ebro Delta from November 2018-June 2020. More studies are needed regarding *M. marinus* prevalence in *C. gigas* in both mortality and non-mortality episodes to understand its role in *C. gigas* mortality

events. Specific challenges in defined shellfish host backgrounds (age class, ploidy, genetic strain, etc.) should also aid in understanding the host-pathogen relationship for this bacterium.

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Authors Contributions

F.U.R. performed experiments, analyzed, and interpreted the experimental data, and drafted the manuscript. F.U.R., K.B.A. and D.M.F. designed the experiments. D.M.F., M.F.T. and M.J.F supervised the study. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

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Supplementary Materials

Supplementary table 1: Sampling data of *Crassostrea gigas* during mortality and non-mortality episodes from 2 bays (Alfacs and Fangar) of Ebro Delta, from November 2018 to June 2020. Sampling size of 30 oysters, except when marked * and ** with 29 and 20 respectively. Enumeration of colonies of morphotypes compatible (like) with: *V. aestuarianus*, *V. splendidus* clade, Other *Arcobacter* spp. and *M. marinus.* Grown on marine agar (MA) at 23 ºC, from pools of 5 homogenate oyster tissue. The Other *Arcobacter* spp. column included the *Arcobacter* spp. other than *M. marinus* as we have mentioned *M. marinus* in a specific column.

A= Alfacs bay; F= Fangar bay; IRTA= grown at IRTA's experimental hatchery, OsHV1 free. D= Diploid; T= Triploid; NA= Data Not Available. A and B represent samples collected at the same time from different rafts. Oysters were grouped into spat (<30 mm), juvenile (30-49 mm) and adult (>50 mm) oysters (Malo et al., 2020) based on shell longitudinal height (mm). S.D.: standard deviation

Sample	Sampling Period	Origin	Age/ Mean Size (mm)	Ploidy	Mortality %	*V. aestuarianus* like average CFU's ±S.D.	*V. splendidus* like clade average CFU's	Other *Arcobacter* spp. like average CFU's	*M. marinus* like average CFU's
1	2018/Nov/29	A	Adult (94)	D	0%	NA	0	0	0
2	2018/Nov/30	F	Adult (79)	D	19%	NA	0	0	0
3	2019/Mar/28-A	IRTA	Juvenile (46)	D	0%	NA	7.1E+02 ±1.2E+02	3.1E+03 ±2.0E+02	0

4	2019/Mar/28-B	A	Juvenile (47)	D	17%	NA	3.6E+02 ±2.9E+01	0	3.6E+03 ±5.0E+02
5	2019/Mar/28-C	F	Juvenile (47)	D	51%	NA	2.2E+03 ±2.7E+02	0	2.2E+04 ±4.7E+03
6	2019/May/24	F	Adult (53)	D	9%	0	3.2E+03 ±3.4E+02	0	1.2E+04 ±1.8E+03
7	2019/May/29	F	Adult (56)	D	0%	0	1.7E+03 ±4.9E+02	9.0E+02 ±1.5E+02	0
8	2019/Jun/27*	F	Spat (23)	D	33%	NA	NA	NA	NA
9	2019/Jun/28-A	F	Adult (84)	D	17%	1.5E+02 ±3.7E+01	0	0	2.3E+04 ±6.3E+03
10	2019/June/28-B	F	Adult (73)	T	51%	2.1E+04 ±7.3E+03	0	0	7.0E+03 ±2.8E+03
11	2020/Feb/25-A**	F	Adult (88)	T	13%	0	6.8E+01 ±8.3E+00	0	5.5E+04 ±9.4E+03
12	2020/Feb/25-B**	F	Adult (80)	T	29%	0	5.2E+02 ±8.2E+01	0	2.5E+04 ±4.6E+03
13	2020/Feb/26	F	Adult (87)	D	0%	0	2.4E+02 ±5.3E+01	1.0E+04 ±4.3E+03	0
14	2020/Apr/21	F	Adult (98)	T	31%	6.3E+03 ±2.0E+03	1.7E+02 ±2.0E+01	0	5.7E+03 ±1.7E+03

15	2020/Apr/22	F	Adult (74)	T	29%	9.6E+03 ±2.7E+03	2.1E+02 ±5.9E+01	0	1.6E+04 ±8.2E+03
16	2020/Jun/04-A**	F	Adult (101)	T	68%	3.4E+05 ±2.9E+04	2.8E+03 ±1.3E+02	0	1.0E+05 ±4.9E+04
17	2020/Jun/04-B	F	Adult (102)	T	37%	0	6.3E+04 ±1.1E+04	0	6.7E+04 ±2.8E+04
18	2020/Jun/17-A	F	Adult (96)	D	7%	0	0	0	9.1E+04 ±2.8E+04
19	2020/June/17-B	F	Adult (99)	D	31%	0	0	0	3.4E+04 ±7.0E+03

Supplementary table 2: Sampling data of *Crassostrea gigas* during mortality and non-mortality episodes from 2 bays (Alfacs and Fangar) of Ebro Delta, from November 2018 to June 2020. Sampling size of 30 oysters, except when marked * and ** with 29 and 20 respectively. Prevalence (%) [prevalence= number of positive individual/total oysters x 100] of OsHV-1, *V aestuarianus, V. splendidus* clade, and *Arcobacter*-like spp. in Pacific oyster's tissue samples (n=30), screened by specific qPCR assays: (Webb et al., 2007; Saulnier et al., 2017; Salas-Massó et al., 2019).

A= Alfacs bay; F= Fangar bay; IRTA= grown at IRTA's experimental hatchery, OsHV1 free. D= Diploid; T= Triploid; NA= Data Not Available. A and B represent samples collected at the same time from different rafts. Oysters were grouped into spat (<30 mm), juvenile (30-49 mm) and adult (>50 mm) oysters (Malo et al., 2020) based on shell longitudinal height (mm).

Sample#	**Time Period**	**Mortality %**	**Origin**	**Age**	**Ploidy**	**OsHV-1 prevalence (%)**	***V. aestuarianus* average prevalence (%)**	***V. splendidus* clade prevalence (%)**	***Arcobacter*-like spp. prevalence (%)**
1	2018/Nov/29	0%	A	Adult	D	0+/30 **(0.0%)**	0+/30 **(0.0%)**	1+/30 **(3.3%)**	0+/30 **(0.0%)**
2	2018/Nov/30	19%	F	Adult	D	0+/30 **(0.0%)**	0+/30 **(0.0%)**	8+/30 **(26.7%)**	2+/30 **(6.7%)**
3	2019/Mar/28-A	0%	IRTA	Juvenile	D	0+/30 **(0.0%)**	0+/30 **(0.0%)**	2+/30 **(6.7%)**	4+/30 **(13.3%)**
4	2019/Mar/28-B	17%	A	Juvenile	D	1+/30 **(3.3%)**	0+/30 **(0.0%)**	11+/30 **(36.7%)**	17+/30 **(56.7%)**
5	2019/Mar/28-C	51%	F	Juvenile	D	16+/30 **(53.3%)**	0+/30 **(0.0%)**	29+/30 **(96.7%)**	5+/30 **(16.7%)**
6	2019/May/24	9%	F	Adult	D	1+/30 **(3.3%)**	1+/30 **(3.3%)**	18+/30 **(60.0%)**	9+/30 **(30.0%)**
7	2019/May/29	0%	F	Adult	D	0+/30 **(0.0%)**	0+/30 **(0.0%)**	24+/30 **(80.0%)**	13+/30 **(43.3%)**
8	2019/Jun/27	33%	F	Spat	D	8+/29 **(27.6%)**	5+/29 **(17.2%)**	2+/29 **(6.9%)**	21+/29 **(72.4%)**
9	2019/Jun/28-A	17%	F	Adult	D	0+/30 **(0.0%)**	0+/30 **(0.0%)**	0+/30 **(0.0%)**	19+/30 **(63.3%)**
10	2019/Jun/28-B	51%	F	Adult	T	0+/30 **(0.0%)**	9+/30 **(30.0%)**	0+/30 **(0.0%)**	22+/30 **(73.3%)**

11	2020/Feb/25-A	13%	F	Adult	T	0+/20 **(0.0%)**	0+/20 **(0.0%)**	12+/20 **(60.0%)**	11+/20 **(55.0%)**
12	2020/Feb/25-B	29%	F	Adult	T	0+/20 **(0.0%)**	0+/20 **(0.0%)**	15+/20 **(75.0%)**	16+/20 **(80.0%)**
13	2020/Feb/26	0%	F	Adult	D	0+/30 **(0.0%)**	0+/30 **(0.0%)**	28+/30 **(93.3%)**	27+/30 **(90.0%)**
14	2020/Apr/21	31%	F	Adult	T	7+/30 **(23.3%)**	4+/30 **(13.3%)**	5+/30 **(16.7%)**	12+/30 **(40.0%)**
15	2020/Apr/22	29%	F	Adult	T	7+/30 **(23.3%)**	3+/30 **(10.0%)**	10+/30 **(33.3%)**	10+/30 **(33.3%)**
16	2020/Jun/04-A	68%	F	Adult	T	15+/20 **(75.0%)**	3+/20 **(15.0%)**	8+/20 **(40.0%)**	16+/20 **(80.0%)**
17	2020/Jun/04-B	37%	F	Adult	T	12+/30 **(40.0%)**	0+/30 **(0.0%)**	7+/30 **(23.3%)**	15+/30 **(50.0%)**
18	2020/Jun/17-A	7%	F	Adult	D	14+/30 **(46.7%)**	0+/30 **(0.0%)**	0+/30 **(0.0%)**	9+/30 **(30.0%)**
19	2020/Jun/17-B	31%	F	Adult	D	6+/30 **(20.0%)**	0+/30 **(0.0%)**	0+/30 **(0.0%)**	18+/30 **(60.0%)**

Supplementary table 3. List of primers used in this study to amplify specific genes or regions for the identification of *Arcobacter*-like spp. and genotypes of the isolates recovered from *C. gigas* and for the detection of putative virulence genes.

PCR	Primer Pair	Function	Sequence of Primers (5´to 3´)	Amplicon Size (bp)	Source
Arcobacter 23S rDNA	23S-F 23S-R	Structure of LSU ribosome	AACATATAAGCGCGATGTGGGGAC ACGGTACGGGCAACATATAATA	233	Salas-Masso et al., 2019; Hausdorf et al., 2013
ERIC PCR (genotyping)	ERIC-1R ERIC-2	Repetitive element in bacterial genomic DNA	ATGTAAGCTCCTGGGGATTCAC AAGTAAGTGACTGGGGTGAGCG	Variable length of DNA fragments	Houf et al., 2002
rpoB	rpoB-Arc15F rpoB-Arc24R	DNA-dependent RNA polymerase subunit B	TCTCAATTTATGGAYCAAAC AGTTATATCCATTCCATGGCAT	845	Salas-Masso et., 2016
***Mma rpoB* (Identification of *Malaciobacter marinus*)**	Mma rpoB-F Mma rpoB-R	DNA-dependent RNA polymerase subunit B	GATTGTAGAGCCTTTAATTGAAG GTTCTAAGAATGGAATAAGTGAG	135	This Study
cadF	*cadF*-F *cadF*-R	Fibronectin-binding protein	TTACTCCTACACCGTAGT AAACTATGCTAACGCTGGTT	283	Douidah et al., 2012
ciaB	*ciaB*-F *ciaB*-R	Campylobacter invasion antigen	TGGGCAGATGTGGATAGAGCTTGGA TAGTGCTGGTCGTCCCACATAAAG	284	Douidah et al., 2012

cj1349	*cj1349*-F *cj1349*-R	Fibronectin-binding proteins	CCAGAAATCACTGGCTTTTGAG GGGCATAAGTTAGATGAGGTTCC	659	Douidah et al., 2012
irgA	*irgA*-F *irgA*-R	Iron-regulated outer membrane protein	TGCAGAGGATACTTGGAGCGTAACT GTATAACCCCATTGATGAGGAGCA	437	Douidah et al., 2012
hecA	*hecA*-F *hecA*-R	One of the filamentous hemagglutinins (FHA) family	GTGGAAGTACAACGATAGCAGGCTC GTCTGTTTTAGTTGCTCTGCACTC	537	Douidah et al., 2012
hecB	*hecB*-F *hecB*-R	Hemolysin activation protein	CTAAACTCTACAAATCGTGC CTTTTGAGTGTTGACCTC	528	Douidah et al., 2012
mviN	*mviN*-F *mviN*-R	Essential protein required for the biosynthesis of peptidoglycan	TGCACTTGTTGCAAAACGGTG TGCTGATGGAGCTTTTACGCAAGC	294	Douidah et al., 2012
pldA	*pldA*-F *pldA*-R	Phospholipase A	TTGACGAGACAATAAGTGCAGC CGTCTTTATCTTTGCTTTCAGGGA	293	Douidah et al., 2012
tlyA	*tlyA*-F *tlyA*-R	Hemolysin	CAAAGTCGAAACAAAGCGACTG TCCACCAGTGCTACTTCCTATA	230	Douidah et al., 2012

3.3 Study 3: First *in vivo* and *in vitro* experimental infections of *Malaciobacter marinus* in adult Pacific oysters (*Crassostrea gigas)* to study its role and the potential synergism with *Vibrio* spp.

(In-preparation)

Summary

The paper describes the study of the potential pathogenic role of some *Arcobacter*-like spp. and *Vibrio aestuarianus* strains isolated in mortality episodes of Pacific oysters (*Crassostrea gigas*) in the Ebro Delta shellfish producing zones, to evaluate its potential implication in such events. The *Arcobacter*-like isolates were genetically characterized to the species level and their potential virulence inferred by the presence of virulence genes followed by performing specific challenge experiments. The potential pathogenicity was investigated for a selection of *Malaciobacter marinus* strains, a specie of *Arcobacter*, isolated only during mortality episodes of *C. gigas* from the Ebro Delta, Spain. The paper also assesses the role of *Vibrio aestuarianus*, which has been reported causing mortalities in adult oysters in the Ebro Delta, Spain and that were isolated simultaneously with the selected *M. marinus*.

During the mortality episodes of Pacific oysters in the Ebro Delta shellfish producing zones, 63.3% of the oyster samples were positive for *Arcobacter*-like spp. by qPCR, while negative for the other known pathogens: Ostreid herpesvirus 1 (OsHV-1), and *Vibrio aestuarianus*. A total of 36 isolates of presumptive *Arcobacter*-like colonies, representing nine different ERIC-PCR genotypes (strains), were identified as isolates of *M. marinus* (26/36, 72.2%) by sequencing of *rpoB* gene. Only 3/26 (11.5%) of *M. marinus* isolates carried three virulence genes: *ciaB, cj1349* and *tlyA*, of the total nine genes studied.

To assess the potential pathogenicity of the *M. marinus* in *C. gigas*, challenge experiments were performed, by injecting the strains in the adductor muscle, a 10% oyster mortality with the strain IRTA-19-131 (harboring no virulence genes) and 25% with IRTA-19-132 harboring three virulence genes). Further cohabitation challenge experiments were carried out with diseased oyster from the challenge with IRTA-19-132 strain as a donor to healthy adults *C. gigas*; and

after three weeks of exposure a 50% and 90% mortality were registered in Cohabitation A (fasting) and Cohabitation B (feeding), respectively. The dead oyster screening from the challenge experiments, reveled that, except for oysters injected with IRTA-19-131 strain; the experimental animals (commercial oysters) also carried *V. aestuarianus* and, therefore, the causality of *M. marinus* in the registered mortalities had to be taken very cautiously. Further challenge experiments (dose-response) with *V. aestuarianus* strains alone and in dual challenge with *M. marinus* showed that mortality increases proportionally with the injected dose in the *V. aestuarianus* challenge, and when a mix infection of both *V. aestuarianus* and *M. marinus* was performed, mortalities were slightly higher in the dual challenge group, however not significantly. To our knowledge, this is the first study where both *M. marinus* and *V. aestuarianus*, isolated during mortality episodes of *C. gigas* in the Ebro Delta, have been tested *in vivo*, to assess its role as potential bacterial pathogens for *C. gigas*. While the pathogenicity of *V. aestuarianus* seems evident, *M. marinus* needs further work with Specific Pathogen Free (SPF) *C. gigas* to discriminate properly among both pathogens.

Furthermore, *in vitro* challenge experiments of *M. marinus* strain IRTA-19-131 with *C. gigas* hemocytes such as Lysosomal membrane stability and bactericidal killing assay, showed that it stressed the *C. gigas* hemocytes and could proliferate in hemocytes, indicating that it has the potential to evade the Pacific oyster immune system.

The study provides insights into the potential role of *M. marinus* in mortality episodes of Pacific oysters (*C. gigas*) and underlines the importance of further studies with *M. marinus* to further elucidate their potential role in the mortality episodes of shellfish, specifically *C. gigas*.

First *in vivo* and *in vitro* experimental infections of *Malaciobacter marinus* in adult Pacific oysters (*Crassostrea gigas)* to study its role and the potential synergism with *Vibrio* spp.

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Abstract

The detection of *Arcobacter*-like spp. during mortality episodes and polymicrobial infections in shellfish raises questions regarding their potential role in such episodes in the Ebro Delta shellfish producing zones, specifically in Pacific oysters *Crassostrea gigas*. The objective of this study was to isolate and characterize *Arcobacter*-like spp. from mortality episodes of *C. gigas* cultures in the Ebro Delta, (Spain) to evaluate its implication in these events. During summer 2019, a 17.5% cumulative mortality was reported by producers in cultured adult diploid Pacific oysters. Animals from the affected area were collected (n = 30) and DNA was extracted to be screened by qPCR for the microvariant genotype of Ostreid herpesvirus 1 (OsHV-1), *Vibrio*

aestuarianus, Vibrio splendidus clade and *Arcobacter*-like spp. Subsequent isolation of *Arcobacter*-like colonies was achieved by enrichment of oyster tissue in two bacteriological media, isolates were genotyped using ERIC-PCR and identified with a 23S rDNA genus-specific qPCR and followed by species level sequencing the *rpoB* gene. Additionally, the presence of nine putative virulence genes was studied in the representative isolates from each ERIC genotype observed. Isolation of *V. aestuarianus* from oyster tissue was performed on marine agar (MA) The oysters collected from the field during mortality episodes were negative by qPCR for OsHV-1, *V. aestuarianus* and *V. splendidus* clade, while 63.3% of the samples were positive for *Arcobacter*-like spp. by qPCR. A total of 36 isolates of presumptive *Arcobacter*-like colonies, representing nine different ERIC-PCR genotypes (strains) were identified with the *rpoB* gene as isolates of *Malaciobacter marinus* (26/36, 72.2%). However, only 3/26 (11.5%) *M. marinus* isolates carried the potential virulence genes *ciaB, cj1349* and *tlyA*. A total of 3/6 (50%) pool samples (i.e., containing 5 oysters each) were positive for *V. aestuarianus*.

Challenge experiments to assess the potential pathogenicity of the *M. marinus* in *C. gigas* were performed by injecting into the adductor muscle of the oysters two strains of *M. marinus* recovered from the mortality episodes: IRTA-19-131 (with no virulence genes) and IRTA-19-132 (with virulence genes). After two weeks, a 10% oyster mortality was observed with the strain IRTA-19-131 and 25% with IRTA-19-132. Two subsequent co-habitation challenge experiments were carried out with diseased oyster of IRTA-19-132 strain as a donor to healthy adults *C. gigas* (fed and fasted). After three weeks of exposure a 50% and 90% mortality were registered in Co-hab A (fasting) and Co-hab B (feeding), respectively.

A dose/ response (lethal dose 50%) challenges were also performed with *V. aestuarianus* (IRTA-19-106 and IRTA-19-107) also isolated from *C. gigas* during the same mortality events in the

Ebro Delta. Since *M. marinus* and *V. aestuarianus* had been isolated simultaneously in mortality episodes, coinfections of both species in *C. gigas* were performed to assess potential synergistic effects. The background bacterial load of the *C. gigas* used for the challenge experiments compromised the interpretation of the *in vivo* experiments. Thus, a series of preliminary *in vitro* challenge assays were performed with *M. marinus*_IRTA-19-131 and *C. gigas* hemocytes and hemolymph.

To our knowledge, this is the first study where both *M. marinus* and *V. aestuarianus*, isolated during mortality episodes of *C. gigas* in the Ebro Delta, have been tested *in vivo*, to assess its role as potential bacterial pathogens for *C. gigas.* While the pathogenicity of *V. aestuarianus* seems evident, *M. marinus* needs further work with Specific Pathogen Free (SPF) *C. gigas* to discriminate properly among both pathogens. Moreover, further, and deeper *in vitro* work with both pathogens separately and in combination could be determinant to assess the role in coinfection of both pathogens.

Key words: *Arcobacter*, *Malaciobacter marinus*, *Vibrio aestuarianus*, virulence, bivalves, Pacific oyster, *Crassostrea gigas*, mortality, challenge, experimental infection.

Introduction

Arcobacter-like species (arc-shaped bacterium) are gram negative, rod shaped, curved, motile and non-spore forming bacteria (Collado and Figueras, 2011; Ramees et al., 2017). They were initially included in the genus *Campylobacter* and were called "aerotolerant campylobacters" (Neil et al., 1979). Later, in 1991, the new genus of "*Arcobacter*" was proposed that was comprised of 4 species namely *Arcobacter butzleri*, *A. cryaerophilus*, *A. skirrowii* and *A. nitrofigilis* (Vandamme et al., 1991; Vandamme et al., 1992b). In the next few years many new species were identified in this genus and now the genus comprises 29 validly published species (Muller et al., 2020). The genus *Arcobacter* was re-classified based on several genome indices and divided into 7 genera namely *Arcobacter*, *Aliiarcobacter*, *Halarcobacter*, *Malaciobacter*, *Pseudarcobacter*, *Poseidonibacter* and Candidate 'Arcomarinus' gen. nov. (Pérez-Cataluña et al., 2018) though this new classification has been challenged (On et al., 2020). *Arcobacter*-like spp. are cosmopolitan in their nature and have been recovered from different hosts and environments which make *Arcobacter* an atypical genus within epsilon Proteobacteria (Debruyne et al., 2008). Lately, *Arcobacter*-like spp. have been isolated in abundance from the marine environment (Levican et al., 2015), abalone (Mizutani et al., 2019) and seafood (Collado and Figueras 2011; Rathlavath et al., 2017; Zhang et al., 2019) such as shellfish (Collado et al., 2009a; Levican et al., 2012; Collado et al., 2014; Mottola et al., 2016), mussels (Figueras et al., 2011), clams, oysters, lobsters (Meziti et al., 2012), seawater (Salas-Massó et al., 2016) and copepods (Gugliandolo et al., 2008).

Due to the recovery of several *Arcobacter*-like species from humans and animal-related infections/ diseases some members of this genus are listed as zoonotic pathogens (Ho et al., 2006; Collado and Figueras 2011; Ramees et al., 2017). *Aliiarcobacter butzleri* is the most

frequently isolated species (Snelling et al., 2006) associated with human diseases, along with *A. cryaerophilus* and *A. skirrowii* (Collado and Figueras 2011; Ferreira et al., 2014; Van den Abeele et al., 2014; Perez-Cataluna et al., 2018), they can cause enteritis, bacteremia (Snellling et al., 2006), septicemia (Lau et al., 2002), and severe diarrhea associated with abdominal pain, nausea and vomiting (Vandenberg et al., 2004; Collado and Figueras 2011; Figueras et al., 2014; Webb et al., 2016). The International Commission on Microbiological Specification for Foods (ICMSF) has included *A. butzleri* in the list of emerging food pathogens representing a serious health hazard for humans (ICMSF, 2002). The mentioned species along with *A. cibarius* and *A. thereius* have been recovered from aborted fetuses of pigs (Schroeder-Tucker et al., 1996; Oliveira et al., 1997; On et al., 2002), cows and goats (Ellis et al., 1977; Snelling et al., 2006; Di Blasio et al., 2019) and can cause enteritis and mastitis (Senelling et al., 2006; Collado and Figueras 2011). Very little knowledge is available on what virulence genes are present in *Arcobacter*-like spp. or virulence mechanisms that are expressed by these species when they infect and colonize the animal and/or human hosts (Snelling et al., 2005b & 2006; Douidah et al., 2012; Mottola et al., 2020).

Worldwide, the total production of oysters in 2019 was 6,259,590 tons, from which 692,317 tons (11.1%) corresponds to the Pacific cupped oyster *Crassostrea gigas* (Thunberg, 1793; FAO, 2021). China, the largest producer in the world, generates 80% of the production, being followed by Korea, Japan, USA, and EU (Dahanayake et al., 2018). Within the European Union, *C. gigas* is the 2nd largest shellfish species by volume, with production of 135,000 tons, accounting for 20% of the total shellfish production, however, it was highest in terms of value and accounted for 45% of the value of total production (EC, 2021). The overall production declined after 2008 when severe *C. gigas* mass mortality events, caused by Ostreid herpes virus type 1 (OsHV-1)

began in Europe (Segarra et al., 2010), and spread globally (Toldra et al., 2019). Other bacterial and viral pathogens have also been involved in mortalities (Paul-Pont et al., 2013; Barbosa-Solomieu et al., 2015; EFSA: EFSA, 2015; Pernet et al., 2016; Lorgeril et al., 2020; Fleury et al., 2020). OsHV-1 mostly affects *C. gigas* spat and juvenile stages causing severe mortality events in Europe every year at a temperature range of 16 ºC to 24 ºC (Prado-Alvarez et al., 2016; Pernet et al., 2012; Renault et al., 2014; Hick et al., 2018). Although with increased age the host can become more resistant (Degremont, 2013; Azema et al., 2016; Pettone et al., 2019). There has been regular detection of OsHV-1 µvar in *C. gigas* in the Ebro Delta with mortalities since 2005 (Roque et al., 2012; Carrasco et al., 2017).

Vibrio aestuarianus is another potent pathogen that causes severe summer mortalities in adult *C. gigas*, but can also affect juvenile oysters (Garcia et al., 2014; Degremont et al., 2014; Barbosa-Solomieu et al., 2015; Fleury et al., 2020). During a 4-year survey in France from 2003-2007, it was found that *V. aestuarianus* and *V. splendidus* clade were the main *Vibrio* spp. detected during the mortality events (Saulnier et al., 2010). Those bacteria have been isolated together with OsHV-1 during mass mortalities of *C. gigas* across Europe (EFSA, 2010). The same occurred in New Zealand during 2010-2011, where *V. splendidus* clade, *V. aestuarianus* and *V. alginolyticus* were found along with OsHV-1 during mortality events (Keeling et al., 2014). Furthermore, *V. aestuarianus* has also been reported causing mortalities in adult *C. gigas* in the Ebro Delta, Spain in 2016-17 (Carrasco et al., 2018).

Several studies have reported that an increase in water temperature changed the composition of the oyster microbiome, and the abundance of *Arcobacter-like* spp. along with known pathogenic *Vibrio* spp. increased with the increased temperature (Green et al., 2019; King et al., 2019). *Arcobacter*-like spp. have also been isolated from the hemolymph of diseased Pacific oyster

(Lokmer & Wegner, 2015) and have been found associated with the spoilage of seafood including *C. gigas* (Madigan et al., 2014; Green et al., 2019). In addition, *Arcobacter*-like spp. have been isolated from oysters and mussels coming from the Ebro Delta growing area in several studies (Collado et al., 2014; Levican et al., 2014; Salas-Massó et al., 2016; 2018) and have been also found in abundance during mortality episodes in *C. gigas*, the virulence potential of these bacteria and their role on the pathogenesis that lead to those mortalities are still unknown (Green et al., 2019). One of the main reasons is the difficulty in isolation of *Arcobacter*-like spp., that requires microaerophilia and enrichment in specific culture media (Rahman et al., 2020). It has been found that during *V. aestuarianus* infection of *C. gigas* the microbiome is dominated by *Vibrio* spp., but also by *Arcobacter*-like spp. resulting in a lower microbial diversity than that found in healthy oysters (Lasa et al., 2019). The detection of *Arcobacter*-like spp. in association with poly-microbial infections highlights the importance of studying the role of these bacterial spp. in relation to the mass mortality events in *C. gigas* in the Ebro Delta. To that aim the present study investigates the pathogenicity of a selection of strains isolated during mortality episodes of *C. gigas* from the Ebro Delta, Spain. Specifically, isolates of *Arcobacter*-like spp. will be genetically characterized to the species level and their virulence potential will be determined by evaluating the presence of virulence genes and performing specific bacterial challenge experiments.

Material and Methods

Oyster Sample Collection

In June 2019, mortality (17.5%) was reported by farmers in adult diploid Pacific oysters in Fangar bay in the north of the Ebro Delta, Spain. Samples of diseased Pacific oysters (*C. gigas*) were collected (40° 46.506'N; 0° 44.246'E), brought to IRTA, La Ràpita, (Spain) where mortality (%) was calculated as number of dead oysters/total oyster sampled x 100. An oyster was marked dead when its open value didn't close upon touching, if the oyster slowly closed the value, it was considered moribund oyster while the oyster who could completely close their value was considered healthy oyster. Environmental parameters (seawater temperature, salinity, and oxygen concentration) during the episode were measured using a SeaBird 19plus (Sea-Bird Scientific, Bellevue, Washington, USA). A total of 30 live oysters were sampled for microbiological culturing, tissue DNA extraction and PCR identification, and for histopathological analysis.

Microbiological culturing

The oysters were washed with tap water and cleaned with a brush to remove residues. Then they were opened with sterile knife and the tissues were collected with sterile scissors and forceps. The whole oyster tissues and inter-valval liquid of those 30 individual oysters were pooled for bacterial culturing. A total of 6 pool samples were made with 5 oysters in each pool. Then pooled oyster samples were weighed and homogenized with an equal amount (w/v) of sterile PBS (Phosphate Buffer Saline) (BD, 257204, Heidelberg, Germany), in a stomacher (Lab·Blender 400, West Sussex, UK) for 2 minutes in sterile double stomacher bags. Later the liquid part of the homogenate was collected in sterile falcon tubes for dilution and plating. The

isolation of *Vibrio* and *Arcobacter*-like spp. were carried out from these tissue homogenates by the method given below.

a. Vibrio spp. isolation

For isolation of *Vibrio* spp. 100 µl of the oyster tissue homogenate, were spread over TCBS agar (thiosulfate citrate bile salts sucrose agar) and MA (marine agar) in triplicates up to 10^{-2} dilution. Then the plates were incubated aerobically at 23 ºC for 24 hours. A representative colony from each distinctive bacterial population was sub-cultured on marine agar and incubated at the same conditions for 24 hours to obtain pure cultures.

b. *Arcobacter*-like spp. isolation

Two enrichment media, Arcobacter broth (Sigma-Aldrich, CM0965, Steinheim, Germany) +CAT [(C.A.T selective supplement: Cefoperazone-Amphotericin B-Teicoplanin) Oxoid™, SR0174E, Hampshire, UK] + 2.5% NaCl (Salas-Massó et al., 2016) and Arcobacter broth +CAT + 50% artificial seawater (Rahman et al., 2020), were used for isolation of *Arcobacter*-like spp. from oyster tissue homogenate samples. As a first step, 1 ml of the oyster homogenates were transferred to 9 ml of these 2 enrichment broth media and incubated aerobically at 23 ºC in static conditions. After 48 hours of incubation, the tubes were vortexed briefly for 10 seconds and 100 µl of the enrichment broth were transferred to 0.45 µm nitrocellulose membrane filters (Millipore, Ref # HAWP04700, Tullagreen, Ireland) overlaid on Marine Agar (MA) (Scharlab, 109352, Sentmenat, Spain). After passive filtration for 30 minutes, the filters were removed, and the plates were incubated aerobically at 23 ºC for 48 hours. The objective of this filtration was to remove the presence of larger non-target bacteria. After incubation, the total colony count and the colony morphology types were recorded. From each MA plate, four colonies of presumably *Arcobacter*-like spp. (i.e., small, transparent/translucent, beige, or off-white colonies) were sub-

cultured individually on MA and incubated in the conditions mentioned above to obtain pure cultures. The colony morphology was recorded and coded, and a loopful was transferred to 180 µl of ATL buffer (Qiagen, Hilden, Germany) for bacterial genomic DNA extraction and pure cultures were kept at the storage temperature of -80 ºC in 20% (v/v) glycerol (Scharlab, GL00271000, Sentmenat, Spain) supplemented marine broth (PanReac AppliChem, C806142, Barcelona, Spain) (Howard 1956) for further work.

DNA Extraction

The oysters were opened with a sterile knife. Approximately 100 mg of tissue from each oyster were homogenized in a bead beater using ~50 mg of 0.5 mm diameter zirconium glass beads (Biospec Products, USA). Then the tissue DNA was extracted using QIAGEN DNeasy® Blood & Tissue Kit (QIAGEN, Ref # 69506, Hilden, Germany). A NanoDrop 2000 (Thermofisher, Wilmington, USA) was used to check DNA concentration. A DNA concentration of 100ng/µl of tissue DNA was used in the different qPCR determinations.

Bacterial qualitative PCR (qPCR)

The DNA from isolated colonies was analyzed for the confirmation of the presumptive *Arcobacter*-like spp. identification by using genus-specific qPCR targeting the 23S rRNA gene for *Arcobacter*-like spp. with SYBR™ Green PCR master mix (Applied Biosystems®, Ref.# 4309155, Woolston Warrington, UK) following the protocol described by Salas-Massó et al. (2019). Oyster tissue DNA was also screened for OsHV-1 using the qPCR assay of Webb et al. (2007) while for *Vibrio splendidus* clade and *V. aestuarianus* the duplex TaqMan qPCR assay of Saulnier et al. (2017) was used.

Genotyping of *Arcobacter*-like spp. by ERIC-PCR

Genotyping of the *Arcobacter*-like isolates was performed in 50µl PCR reactions with ERIC 1R and ERIC 2 primers (Versalovic et al., 1991) to avoid working with multiple redundant clones of the same strain and species. A total of 5µl of amplified ERIC-PCR product was run through a 2% agarose gel for 2 hours using 100 volts, and the resulting bands were compared to those of the 1kb plus ladder molecular weight standard (Invitrogen^{TM}, Ref.# 10787-018, Carlsbad, USA). The band patterns of the ERIC-PCR products of all isolates were compared to each other for the purpose of screening and identifying clones/strains of the same species. Genotype designations were assigned as DE-1 through DE-8 upon visualization of the distinct banding patterns obtained (Figure 1). If there was a difference of one or more bands, they were considered different genotypes (Levican et al., 2013a) and analyzed further by *rpoB* gene sequencing.

***rpoB* gene amplification, sequencing, and phylogenetic analysis of *Arcobacter*-like isolates**

Representatives of each of the different ERIC genotypes were identified to the species level using the sequence of the *rpoB* gene, as described earlier (Collado et al., 2009b; Salas-Massó et al., 2016). Amplification of the *rpoB* gene was performed using 50µl reactions which included 48-µl that contained 5µl reaction buffer (10X), 4µl of $MgCl_2$ (50 mM), 4µl of dNTP's (10 mM), 0.2µl of Taq DNA polymerase (5 U/µl); (Invitrogen, Ref.# 10342-020, Carlsbad, USA), 1µl forward primer and 1µl reverse primer (20 µM each), and 32.8µl of sterile ultrapure distilled water (Invitrogen, Ref.# 10977-035, Paisley, UK). The amplified product was analyzed on a 1.5% agarose gel, then the PCR product was purified using QIAquick® PCR purification kit (QIAGEN, Ref.# 28104, Hilden, Germany). The purified product was sequenced commercially (Sistemas Genomicos, Valencia, Spain) using the same primers as those employed in the amplification. A consensus sequence derived from forward and reverse reads of each isolate was

used for BLAST analysis in the NCBI database to check for the similarity index with existing *Arcobacter*-like spp. in the database. The sequences from high scoring BLAST hits, together with the obtained *rpoB* sequences, were aligned using Clustal W in BioEdit 7.2.5 (BioEdit Sequence Alignment Editor, Hall 1999). The aligned sequences were trimmed to the same length (425 bp) and a phylogenetic tree was constructed by Neighbor-Joining (NJ) method including 1000 boot strap replicates using the Kimura 2-parameter model in MEGA 6 software (MEGA, Tamura et al., 2013). The isolates that clustered as novel isolates in the *rpoB* gene phylogenetic tree were further classified by amplification and sequencing of *atpA* and *gyrB* gene (Supplementary table 1) with the same PCR conditions of *rpoB*. The consensus sequences were built from both forward and reverse sequences of both these genes and the consensus sequence used for searching for similarities in the NCBI database and phylogenetic analyses were performed for both these genes as discussed above for the *rpoB* gene. Later, concatenated sequences (1602 bp) were built from the *atpA* (563 bp), *gyrB* (614 bp), and *rpoB* (425 bp) genes sequences of the potential novel isolates and similar sequences retrieved from NCBI's nucleotide database. Then a phylogenetic tree was constructed with Neighbor-Joining (NJ) method including 1000 boot strap replicates using the Kimura 2-parameter model in MEGA 6 software as discussed above.

Presence of putative virulence genes in *Arcobacter*-like isolates

The *Arcobacter*-like isolates were screened for the presence of nine putative virulence genes i.e., *cj1349, cadF, hecA, irgA, hecB, mviN, pldA, tlyA* and *ciaB,* using the primers (Supplementary table 1) and conditions described in previous studies (Douidah et al., 2012; Collado et al., 2014). *A. butzleri* LMG 10828^{T} DNA was used as a positive control as it carries nine putative virulence genes in its genome (Douidah et al., 2012).

For the primer sets *ciaB, cj1349, hecA, irgA* and *mviN* the reaction mixture (50µl final volume) contained 2µl of DNA extract (50 ng/µl), 1.5 U of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA), 0.2 mM each deoxyribonucleotide triphosphate (dNTP's), 50 pmol of each primer, 5µl of 10X PCR buffer (Invitrogen, CA), and 1.3 mM $MgCl_2$. For the primer sets *cadF, hecB, pldA* and *tlyA* reaction ingredients were the same but contained 1.5 mM $MgCl_2$. Amplified products were detected by electrophoresis in 1.5% agarose in 0.5 Tris-acetate-EDTA (TAE) buffer at 100 V for 60 min. Gels were stained with ethidium bromide. A gel documentation system (GeneFlash, Syngene Bioimaging, Cambridge, UK) was used for visualization.

In vivo challenge experiments of *C. gigas*

Pacific Oyster (*Crassostrea gigas*) baseline screening and challenge with *M. marinus* strains and/or *Vibrio aestuarianus*

A series of experimental infections were performed with both *M. marinus* and *V. aestuarianu*s strains isolated during *C. gigas* mortality episodes in the Ebro Delta shellfish production zones. Adult *C. gigas* oysters grown in Fangar Bay, were bought from a local shellfish depuration plant for the experimental challenges with *M. marinus and V. aestuarianus*. Shell size (length and width) were measured, and 15 animals were sacrificed 24 hours prior to a challenge and screened for the presence of prevalent *C. gigas* pathogens endemic to the area i.e., OsHV-1, *V. aestuarianus, V. splendidus* clade, and *Arcobacter*-like spp. The *C. gigas* tissues were analyzed both by PCR after extracting the DNA and by culturing, as described above. The remaining animals were held for 24 hrs. to acclimatize to the ambient conditions of the experimental aquaria at 24 °C ±2. The animals were checked twice a day for sickness and mortality. Generally, when an animal did not close its valves quickly (in 10 seconds after touching), or valves closed halfway or not tightly closed, and were slow in response, they were considered

moribund (Azema et al., 2016). From the animals found moribund during the challenge, the tissue samples were collected for DNA extraction and the remaining tissue was homogenized for bacterial isolation as discussed above.

Inoculum Preparation

Two *M. marinus* strains i.e., strain IRTA-19-131 (with no detected putative virulence genes) and strain IRTA-19-132 (having detected three putative virulence genes i.e., *ciaB, mviN* and *tlyA*) were selected for the challenge experiments in *C. gigas.* Initially, preliminary Optical Density (OD) measures vs CFU correlations were established for the *M. marinus* strains IRTA-19-131 and IRTA-19-132, using cell suspensions from 24 hours fresh culture grown on marine agar (MA) with OD_{420} of 0.4, 0.6, 0.8 and 1.0 measured using a photometer (Model D- OD 100, Dinko instruments, Spain). Each suspension was 10-fold serially diluted and 100µl from each dilution was plated on marine agar in triplicates and incubated aerobically for 48 hours at 23 ºC. During the same mortality episode of June-2019 from adult Pacific oysters from Fangar bay two *V. aestuarianus* strains (IRTA-19-106 and IRTA-19-107) were isolated. A similar procedure, as the one described above, was followed to prepare the inoculum for the *V. aestuarianus* strains (IRTA-19-106 and IRTA-19-107).

For the challenge by injection experiments, after acclimatization, animals were anesthetized in a solution of $MgCl_2.6H_2O$ (Acros Organics™, Cat#197530250, Geel, Belgium) [50 g/l of 1:4 seawater: $d.H_2O$] for 3 hours.

Experiment 1: Bacterial challenge by injection

The challenge experiments were performed in a bio-confinement room, in 20-liter glass aquariums holding 15-liter of UV filtered seawater at 24 ºC ± 2 with air supply unless otherwise

stated. The number of animals in each aquarium was ten and a total of 10 aquariums for 5 conditions in duplicate were set up (Supplementary figure 1). The experimental conditions involved 1) the negative/negative control (NNC) animals were not exposed to anesthesia solution nor to pathogens; 2) Anesthesia control (AC) animals, using the protocol described by Suquet et al. (2009) were only exposed to anesthesia solution (mentioned above); 3) animals were exposed to anesthesia and injected with 0.1 ml of 50% sterilized artificial seawater (SASW); 4) MS1:anesthetized and injected oysters with 0.1 ml 10^8 CFU/ml of *M. marinus* strain 1 (IRTA-19-131) and 5) MS2: oysters anesthetized and injected with 0.1 ml of 10^8 CFU/ml of *M. marinus* strain 2 (IRTA-19-132).

After injection, the animals were kept dry for 15 minutes, to facilitate inoculum internalization within the oysters before transferring them to the water tanks. The water in the tanks was renewed daily, and animals were not fed throughout 21 days of the experiment. During the challenge, animals were checked twice a day to collect moribund/dead individuals. The animals were checked individually for their valve closure response and moribund/sick oyster were sampled as commented above.

Experiment 2: Bacterial challenge by cohabitation

Having observed moribund animals (n= 2) that had been injected with the *M. marinus* strains, 72 hours after the experimental challenge by injection, a new challenge experiment was initiated as follows. Two glass tanks of 20 L capacity, with 15 L of UV, filtered, aerated sea water at 24 ºC, were set up to hold 10 adult oysters in each, as described above. Then, two moribund oysters from the injection challenge experiment MS1 with *M. marinus* strain IRTA-19-132 were transferred, one to each tank to a ratio of 1/10 (moribund/healthy) oysters, and cohabitation of the moribund injected oysters (donors) with asymptomatic receivers (healthy and a new oyster

group) was carried out for 24 hours. Following the exposure of 24 h, the moribund oysters were removed from the tanks and were processed for tissue DNA extraction to screen for OsHV-1 and bacterial pathogens (i.e., *Vibrio aestuarianus, V. splendidus* clade and *Arcobacter*-like spp.) by qPCR assay and bacterial culturing as explained above and the presence of *M. marinus* by qPCR assay (publication in preparation). Moreover, the cohabitation challenge was run with two different conditions: with feed and fasted oysters. The oysters in tank A were given 1 L of phytoplankton mixture [*Isochrysis galbana* (Parke, 1949), *Chaetoceros neogracilis* (Van Landingham, 1968), *Tetraselmis suecica* (Kylin) (Butcher, 1959) with the following proportions of 60:30:10, respectively] with a cell density of 60-80,000 cells/ml, but in tank B the oysters used were fasted. The cohabitation experiment was carried out for 15 days, the oysters were observed daily, and any moribund/ dead oyster was processed as described above.

Experiment 3: *Crassostrea gigas* challenge with *Vibrio aestuarianus*

The *V. aestuarianus* strains (IRTA-19-106 and IRTA-19-107) co-isolated with *M. marinus* from the same oyster samples (Table 1), were grown on MA during 24 hours at 23 ºC. Then a 0.1 ml of the latter strain suspensions at three concentrations (10^8, 10^7 and 10^6 CFU/ml) were injected into the adductor muscles of adult Pacific oysters (n=5 per group). The animals were not fed during the experiments and were observed for 15 days while being exposed to the same environmental conditions as mentioned in the *M. marinus* challenge experiment above.

Experiment 4. Dual challenge of oyster with *M. marinus* and *V. aestuarianus* strains

After the individual challenge experiments, a dual challenge experiment was planned to find out the combined effect of both *M. marinus* strain IRTA-10-131 or IRTA-19-132 and *V. aestuarianus* IRTA-19-106. The inoculum concentration of 10^7 cfu/ml for *V. aestuarianus* IRTA-19-106 and 10^8 cfu/ml for both *M. marinus* strains were prepared in 50% SASW. Then 0.1

ml of each bacterial suspension of *M. marinus* IRTA-19-131 (MS1) and IRTA-19-132 (MS2) and *V. aestuarianus* IRTA-19-106 (VA6) were each injected separately into 3 groups of oysters (n = 20 each group) that were later divided into 2 groups for duplicate testing (n = 10 x 2). While for the dual challenge, a group of 20 oysters were injected with 0.1 ml of *V. aestuarianus* IRTA-19-106 and 0.1 ml of *M. marinus* IRTA-19-131 (MV316), while another group of 20 oysters were injected with same dose of *Vibrio* strain IRTA-19-106 and *M. marinus* IRTA-19-132 (MV326). The control (SASW) was injected with the same amount of 50% SASW while another negative-negative control (NC) was only exposed to anesthesia solution. The animals were exposed to the same environmental conditions as for the above challenge experiments and were observed for 15 days for mortality.

Histopathological examination

Before starting the challenge samples from healthy oysters were sampled for histological analysis to establish a baseline for any tissue response that might be observed while the oysters were sampled during the challenge after injection with *M. marinus* strain in experiment MS1. For preparation, oysters were opened with sterile knife to remove their body from the shell. A transversal cross-section of the oyster body was made (Avila-Poveda et al., 2014) and thereafter the two parts were immersed for 24 hours in Davidson's fixative solution (Shaw and Battle 1957). They were then kept in 70% ethanol until they were processed using an automatic tissue processor to embed them in paraffin blocks, from which 5 μm sections were cut with the microtome (HistoCore BIOCUT, 149BIO000C1, Leica Biosystems, Nussloch GmbH). Then sections were deparaffinized, re-hydrated and stained with hematoxylin and eosin (H&E) (Gomez-Leon et al., 2008). The histopathological examination of the samples was carried out using a light microscope (Leica DMLB/ LB30T, Leica Microsystems, Wetzlar, Germany)

equipped with an Olympus DP70 digital camera (Olympus Corporation, Tokyo, Japan) and a DP Controller v2.1.1.183 software (Olympus Corporation).

In vitro experiments with *C. gigas* hemocytes

Bacterial culture

The above-mentioned train IRTA-19-131 from *M. marinus* was cultured on marine agar for 48 hours at 23 ºC in aerobic condition. Then a loopful of the culture was transferred to 50ml of Arcobacter broth + 50% ASW and was incubated overnight, aerobically in a shaker incubator at 100 rpm at 23 ºC. The bacterial cells were harvested by centrifugation at 4500 x g for 20 min. The supernatant was discarded, and the pellet was re-suspended in 1 ml 50% SASW. A fresh inoculum was prepared for each experiment by adjusting the $OD_{420\ nm}$ to 0.4 in a new vial of 50% SASW.

Animals and hemolymph collection

Adult *C. gigas*, 7-10 cm long, were purchased from local depuration plant in Ebro Delta, Spain. The animals were kept in aerated tanks to acclimatize before the experiments. Hemolymph was extracted from the posterior adductor muscle using a sterile 1 ml syringe with a 21-G needle. The needle was removed, and hemolymph was filtered through a sterile gauze and pooled in 50ml Falcon tubes at 16 ºC by the method described earlier (Auguste et al., 2022).

Lysosomal membrane stability (LMS)/ Neutral red retention time (NRRT) assay

The test was conducted on isolated oyster hemocyte cells as described earlier (Auguste et al., 2022). The extraction was carried out from 5 animals for a single treatment, and 20µl were placed on 4 microscope slides (Each slide for a concentration of bacterial suspension i.e., 10^6,

10^7, and 10^8 CFU/ml and one as a control slide) inside a humid chamber and the cells were allowed to attach to the slide at 16 °C for 20 minutes. For the control slide 20µl of 50% SASW was placed onto the slide. The excess bacterial suspension and 50% SASW was removed from the slides and 20µl of a dilute solution of neutral red (5µl solution stock (28.8 mg/ml DMSO) + 995µl seawater) was placed on the slides and incubated for 15 min. After removing the neutral red 20µl of 50% SASW was added to the slides that were closed with coverslips and sealed with enamel and observed at 15 minutes intervals using an optical microscope under a 40X magnification (Leica Microsystems, DM500, Germany).The illumination level was kept to a tolerable minimum during observations and the time at which it was evident the release of dye from the lysosomes in the cytosol due to destabilization of the lysosomal membranes. The test for each slide was conducted in 10 fields of observation and about 10 cells were evaluated for each field. The endpoint was calculated as the time in which in 50% of the observed fields the neutral red was released by the lysosomes, or the membranes were destabilized.

Bactericidal killing assay

For assessing the ability of *C. gigas* hemocytes for killing of *M. marinus* strains IRTA-19-131 three conditions were used a) hemolymph only, as negative control b) *M. marinus* strain IRTA-19-131 + hemocytes and c) *M. marinus* strain IRTA-19-131 suspension (at a concentration 1-2 x 10^6 CFU/ml). Each condition had 3 time points: T0 minutes, T30 min and T90 min which correspond to the time that they were incubated in the wells of multi-well plate. The hemocytes monolayer was prepared by collecting hemolymph from adductor muscle as described above. Then 500µl of hemolymph was added into each well of a 24 well plate using 2 conditions a) and b) and all 3 time points and incubated for 40 min at 16 °C. The supernatant was removed (without touching the monolayer), and the wells were washed using 500µl of 50% SASW. From

the *M. marinus* strain IRTA-19-131 suspension, 500µl was added to each well of condition b) and c) and incubated for the three time points T0, T30 and T90 min. At the end of each time point, the hemocyte monolayer was broken with the pipette tip and all content of the wells were collected individually in tubes containing 4ml of ASW + triton 0.05%. Each well was then washed one time with 500µl ASW + triton 0.05% solution and collected in the same tube used before making a final volume of 5ml (4 ml of the beginning + 0.5 from the well + 0.5 from the well washing). The tubes were properly mixed by vortexing and 100µl of the mix were used for serial dilutions 1:10 (100µl + 900µl ASW), plated in triplicates on marine agar and incubated aerobically at 23 ºC for 48 hours. Thereafter the growing colonies were counted to calculate the CFU/ml. The percentage of killing was calculated for T30 and T90 with respect to T0 by the formula [% killing= 100- (CFU/ml of respective time point X 100/CFU/ml of T0)].

Statistical analysis

The biometry of the *C. gigas* samples, as well as the mean and standard deviation of the duplicate values of the qPCR results was calculated in MS Excel 2016. The performance of the two-enrichment broth i.e., Arcobacter-CAT broth + 2.5% NaCl (Salas-Massó et al., 2016) and Arcobacter-CAT broth + 50% ASW (Rahman et al., 2020) used for recovery of *Arcobacter*-like spp. was analyzed evaluating the growth abundance and bacterial diversity. The latter results were compared by T-test in IBM SPSS Statistics package (Version 21.0) for Windows (IBM Corp, Armonk, NY, USA). The survival analysis of the *C. gigas* in the bacterial challenge experiments with *M. marinus* and/or *V. aestuarianus* was assessed using the Kaplan-Meier survival analysis in Sigma Plot v 12. The significant difference (*p* value) was calculated using a standard cutoff value of 0.05. The results of the *in vitro* hemocytes experiments are the mean and standard deviation (SD) of at least 5 experiments and analyses, unless otherwise indicated,

performed in duplicate and triplicate. Statistical analysis was performed by comparing the means of the different treatment groups by pairwise T test in SPSS v21 (IBM SPSS Statistics 21). The statistical analysis at 95% confidence ($p < 0.05$) was considered significant.

Results

Oyster sampling and pathogen detection in tissue DNA by qPCR assays from natural mortality episode in June 2019 (Fangar bay)

The water temperature in the Fangar Bay at the time of sampling was 26.1 °C, practical salinity (S_P) was 34, and 6.7 mg/L oxygen concentration. The average size of the adult oyster samples collected (n = 30) during the June-2019 mortality from Fangar bay, Ebro Delta was 84.2 ± 9.18 mm, and the calculated cumulative mortality was 17.5% [11/63 x 100 (dead oyster/total oyster sampled x 100)]. There was no discoloration, cysts, or any other visible sign of pathology (Carvalho-Saucedo et al., 2019) in the oyster tissue.

qPCR analyses and Isolation of *Vibrio* spp. and *Arcobacter*-like isolates from a natural mortality episode in June 2019 (Fangar bay)

None of the 30 oysters tissue samples collected from the mortality episode were positive for OsHV-1, *V. aestuarianus* and *V. splendidus* clade by qPCR analysis, while 19/30 (63%) samples were positive for *Arcobacter*-like spp. by 23S genus-specific qPCR assay (Table 1).

Table 1: Results of pathogen detection by qPCR from the tissue DNA of 30 individual adult diploid Pacific oysters (*Crassostrea gigas*) collected during summer mortality of June 2019 from

Fangar bay of Ebro Delta, Spain. The bacterial isolation on marine agar is represented as average CFU/ml of the six oyster tissue pools containing 5 oysters in each pool (6 pools x 5 oyster each) and incubated aerobically at 23 °C. ND = not detected.

Oyster' s Average Size (mm)	**Mortality (%)**	**Matrix**	**OsHV-1**	***Vibrio aestuarianus***	***Vibrio splendidus* clade**	***Arcobacter*-like spp.**
84.2 ± 9.18	17.5	Tissue qPCR detection	ND	ND	ND	19 (63%)
		Average number of bacteria by culture CFU/ml	___	Cultured on Marine agar. 1.48E+02 ±3.73E+01	Cultured on TCBS and Marine agar. 0	Cultured on Marine agar. 2.31E+04 ±6.30E+03

A confluent growth of *Vibrio* spp. colonies was observed (n >300) on TCBS media from the 6 pool samples (5 oysters in each pool) after 24 hours of aerobic incubation at 23 °C. The growth abundance and diversity were similar for all 6 pools. Yellow, large (3-5 mm in diameter) and opaque colonies were very abundant (n = >300) on TCBS agar. Also, green, opaque colonies grew (3-5 mm in diameter) but were less abundantly present (n = <50). The Vibrio Duplex TaqMan™ qPCR assay performed with 30 selected colonies of both main types (yellow and

green), did not show any positive results for *V. splendidus*-like species in the samples collected from mortality episodes.

On Marine agar (MA), there was growth of small (0.5 to 1mm), white, circular, translucent colonies from oyster tissue homogenate collected during mortality events. They were present in low abundance (n = <50 colonies) in 3/6 pool samples: pool #3, pool #4 and pool #5 (Table 1). The three representative isolates from these pools were identified as *V. aestuarianus* by Vibrio duplex TaqMan^{TM} qPCR assay. The average CFU/ml of these *V. aestuarianus* isolates from these pool samples are given in table 1.

Characteristic small (approximately 1mm in diameter), beige colonies of *Arcobacter*-like spp. grew on MA plates after 48 hours of aerobic incubation at 23 ºC following two enrichment steps in broths i.e., Arcobacter-CAT broth + 2.5% NaCl and Arcobacter-CAT broth + 50% ASW. Interestingly, from Arcobacter-CAT + 2.5% NaCl enrichment broth only 3/6 (50%) pool samples showed growth on MA, while with Arcobacter-CAT + 50% ASW enrichment broth, 6/6 (100%) of pool samples showed growth of *Arcobacter*-like spp. on MA. A total of 12 and 24 isolates were selected and sub-cultured from Arcobacter-CAT broth + 2.5% NaCl and Arcobacter-CAT + 50% ASW enrichment broths, respectively. From these, 4 morphologically similar colonies from each pool were selected for further study. All these isolates were gram negative, curved rods, catalase, and oxidase positive. The extracted DNA of all 36 isolates confirmed the presence of *Arcobacter*-like cells using the Arcobacter 23S rDNA qPCR assay. The average CFU/ml of *Arcobacter*-like spp. from is given in table 1.

Genotyping by ERIC-PCR of *Arcobacter*-like isolates recovered from the June 2019 mortality episode

The ERIC-PCR patterns of 36 *Arcobacter*-like isolates recovered from the two enrichment broth media i.e., Arcobacter-CAT broth + 50% ASW and Arcobacter-CAT broth + 2.5% NaCl are shown in figure 1 and table 2.

The 24 isolates recovered from Arcobacter-CAT broth + 50% ASW, resulted in a total of nine genotypes (DE-1 to DE-9). The genotype DE-3 (representative isolate was IRTA-19-147) was the most abundant genotype, 5/24 isolates (20.83%), recovered from this enrichment broth. While genotypes DE-1 (IRTA-19-131) and DE-8 (IRTA-19-137) was the least abundant genotypes recovered from Arcobacter-CAT broth + 50% ASW enrichment broth. Both DE-1 and DE-8 genotypes consisted of only one isolate (1/24 = 4.17% each) recovered from this enrichment broth. The 12 *Arcobacter*-like isolates recovered from Arcobacter-CAT broth + 2.5% NaCl represented only two genotypes DE-7 (3/12 isolates, 25%) and DE-8 (9/12, 75%). These genotypes were also recovered from Arcobacter-CAT broth + 50% ASW (Figure 1 and Table 2). Seven genotypes (DE-1, DE-3 to DE-6 and DE-7) were only isolated with Arcobacter-CAT broth + 50% ASW enrichment broth.

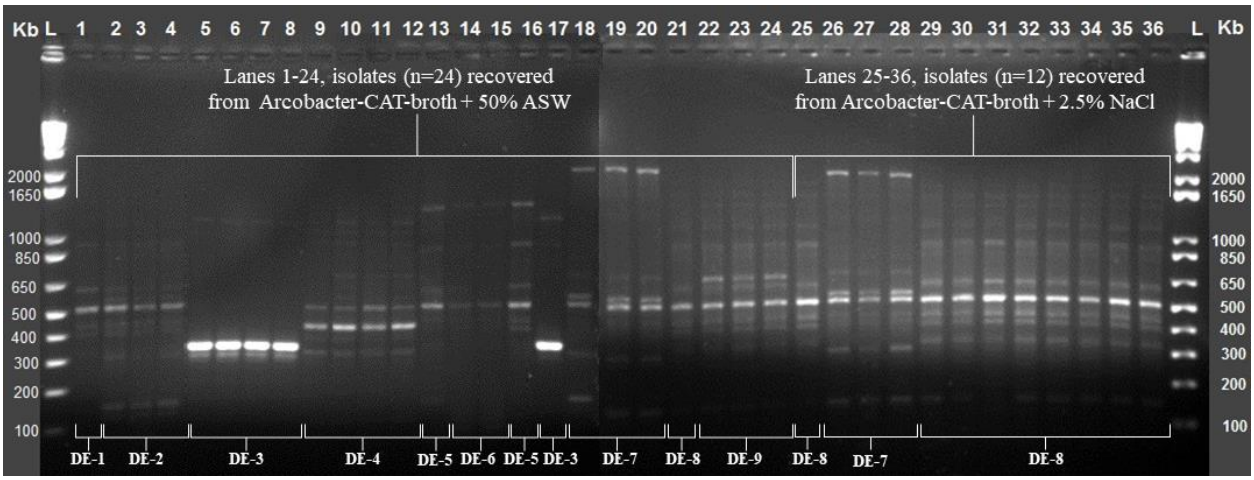


Figure 1. ERIC-PCR banding pattern of *Arcobacter*-like isolates recovered from oysters during a mortality event in Fangar bay June 2019. L = 1 kb plus ladder (Invitrogen™, 10787018); The numbers above (lanes) represent each of the 36 genotyped isolates (n = 24 or n = 12 recovered from different enrichment media). The different identified ERIC genotypes correspond to lane 1= genotype DE-1; lanes 2-4= genotype DE-2; lanes 5-8 and 17= genotype DE-3; lanes 9-12= genotype DE-4; lanes 13 and 16= genotype DE-5; lanes 14 and 15= genotype DE-6; lanes 18-20 and 26-28= genotype DE-7; lanes21, 25 and 29-36= genotype DE-8 and lanes 22-24= genotype DE-9.

Table 2: Abundance (Number of isolates) of *Arcobacter*-like genotypes isolated on marine agar at 23 ºC under aerobic condition from adult diploid Pacific oysters recovered from two enrichment broth media i.e., Arcobacter-CAT broth + 50% ASW and Arcobacter-CAT broth + 2.5% NaCl. CAT= Cefoperazone, Amphotericin B and Teicoplanin supplement; ASW= Artificial Seawater. The identification of these *Arcobacter*-like isolates was carried out by amplification and sequencing of the *rpoB* gene and confirmed by *M. marinus*-specific qPCR.

Genotype Identified by ERIC	**Representative Isolate**	**Number of Isolates**	**Number of isolates from Arcobacter-CAT-broth + 50% ASW**	**Number of isolates from Arcobacter-CAT-broth + 2.5% NaCl**	**Mma rpoB qPCR result from the genotype**	**Identification by *rpoB* gene sequencing**
DE-1	IRTA-19-131	1	1	0	+	*M. marinus*
DE-2	IRTA-19-132	3	3	0	+	*M. marinus*
DE-3	IRTA-19-147	5	5	0	+	*M. marinus*
DE-4	IRTA-19-133	4	4	0	-	Potential novel *Arcobacter*-like spp.
DE-5	IRTA-19-134	2	2	0	+	*M. marinus*
DE-6	IRTA-19-135	2	2	0	+	*M. marinus*
DE-7	IRTA-19-136	6	3	3	-	Potential novel *Arcobacter*-like spp.
DE-8	IRTA-19-137	10	1	9	+	*M. marinus*
DE-9	IRTA-19-138	3	3	0	+	*M. marinus*
Total		36	24	12	7/9	

rpoB gene sequencing and phylogenetic analysis

The BLAST analysis in the NCBI nucleotide database of consensus sequences of *rpoB* genes from nine representative isolates (Genotype DE-1, to DE-9) showed that majority of the obtained genotypes (7/9, 78%), representing 72% (26/36) of *Arcobacter*-like isolates recovered from the natural mortality episode of oysters in June 2019, showed similarity with *M. marinus* strains

(Figure 2). The isolates IRTA-19-131, IRTA-19-147, IRTA-19-134, IRTA-19-135, IRTA-19-137 and IRTA-19-138 showed 99.6-100% similarity with the *rpoB* gene sequence from *M. marinus,* except the DE-2 genotype (strain IRTA-19-132) that showed 96.97% similarity with *M. neptunis* (Accession No. LT904787.1). Genotype DE-3 comprised 4/36 (11%) isolates and was represented by strain IRTA-19-147 that show a 97.2% similarity of the *rpoB* gene sequence with *Arcobacter* sp. F155-44 (Accession No. LT904793.1). Genotype DE-7 corresponded to 6/36 (16.7%) of isolates and the representative strain IRTA-19-136 showed 98.6% similarity with the *rpoB* gene sequence of *Arcobacter* sp. strain F146-38 (Accession No. LT904787.1). The Neighbor-Joining (NJ) phylogenetic tree of the *rpoB* gene sequences shows that of 7/9 representative genotypes, corresponding to 78% of isolates, are grouped together with *Malaciobacter marinus* (Figure 2). The Neighbor-joining phylogenetic tree (Supplementary figure 2) from the concatenated sequences (1602bp) of *atpA* (563bp), *gyrB* (614bp), and *rpoB* (425bp) of the potential novel isolates i.e., genotypes DE-2 (IRTA-19-132), DE-4 (IRTA-19-133) and DE-7 (IRTA-19-136) strains showed that DE-2 (IRTA-19-132) was a *M. marinus* strain. However, the other two genotypes DE-4 (IRTA-19-133) and DE-7 (IRTA-19-136) were potential novel isolates.

RESULTS AND DISCUSSION

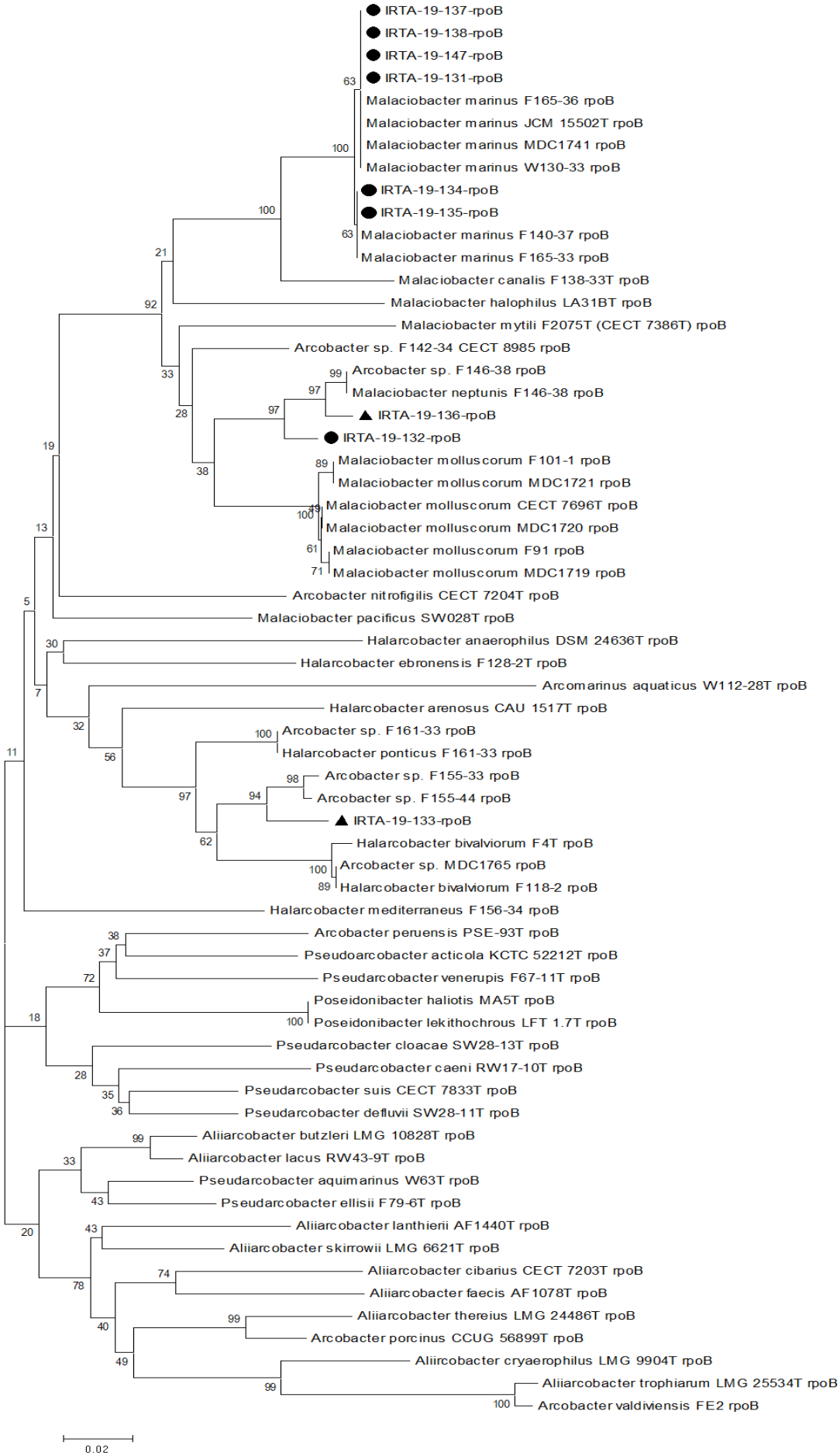


Figure 2. Neighbor-Joining phylogenetic tree showing the relationship of the *Arcobacter*-like strains recovered from the mortality episode of adult Pacific oysters based on the *rpoB* gene (425 bp). (Bootstrap percentage values based on 1,000 replications are shown at the nodes of the tree.) Bar indicates two substitutions per 100 nucleotides.

Presence of putative virulence genes in *Arcobacter*-like strains

The PCR results for detecting the presence of nine putative virulence genes in the *Arcobacter*-like strains showed that all except IRTA-19-132 and IRTA-19-136 (isolates of genotypes DE-2 and DE-7, respectively), showed a negative detection for the putative virulence genes evaluated (Table 3). Strain IRTA-19-132 carried three putative virulence genes (i.e., *ciaB,* that encode for a *Campylobacter* invasion antigen, the *cj1349* gene that helps in the adhesion to cell surfaces, and *tlyA* that encodes for a hemolysin). However, strain IRTA-19-136 carried only the *ciaB* and *tlyA* genes.

Table 3: Presence/absence of nine putative virulence genes from *Arcobacter*-like strains isolated from diploid adult Pacific oysters during a mortality event in June-2019. *Aliiarcobacter butzleri* LMG 10828^{T} was used for a positive control as it carries all nine of the putative virulence genes.

Representative *M. marinus* Isolate	**Virulence genes studied**								
	ciaB	***cj1349***	***hecA***	***irgA***	***mviN***	***cadF***	***hecB***	***pldA***	***tlyA***
IRTA-19-132	+	+	-	-	-	-	-	-	+
IRTA-19-136	+	-	-	-	-	-	-	-	+

IRTA-19-131, IRTA-19-133, IRTA-19-134, IRTA-19-135, IRTA-19-137, IRTA-19-138, IRTA-19-147	-	-	-	-	-	-	-	-	-
Aliiarcobacter butzleri LMG 10828^T	+	+	+	+	+	+	+	+	+

Pacific oysters challenged with two *M. marinus* and *V. aestuarianus* strains recovered from the oyster mortality episode of June 2019

OD vs CFU correlation for *M. marinus* inoculum and *V. aestuarianus*

The OD_{420} vs CFU/mL correlation showed that cell suspension with $OD_{420\ nm}$ = 0.8 corresponds to 10^8 CFU/mL for both *M. marinus* strains IRTA-19-131 and IRTA-19-132, which was used for preparing the inoculum in a 1:10 dilution cascade. In the case of *V. aestuarianus*, the correlation of 0.5 $OD_{550\ nm}$ corresponded with 10^8 CFU/mL, for both strains A (IRTA-19-106) and B (IRTA-19-107).

Baseline pathogen screening prior to oyster challenge

A prior screening of the Fangar bay oysters (purchased locally from depuration plant) using the specific qPCR for OsHV-1 and for the *V. aestuarianus* and *V. splendidus*-clade was performed using 15 individual oysters tissue DNA samples, and all of them showed negative results

(Supplementary table 2). However, in nine of the 15 oysters (60%) there were the presence of *Arcobacter*-like spp. was detected using a PCR targeting the 23S rRNA gene, but none of the samples of oyster tissue reacted to the specific *M. marinus* qPCR (the assay is described in another unpublished manuscript) targeting the *rpoB* gene of this species. In the case of bacterial culturing of three pools (each of them containing homogenized samples from five oyster tissue), one pool was positive for *V. aestuarianus* with 1.2 x 10^4 CFU/mL (Supplementary table 3).

Experiment 1: Bacterial challenge by injection of the selected bacteria into the adductor muscle

There was a total of two oysters dead out of the 20 tested (10%) in the MS1 oyster group challenged with 1.8 X 10^8 CFU/mL of *M. marinus* (IRTA-19-131) within 48 hours post-injection (hpi) despite animals being observed for 21 days post-injection (dpi). In the case of the MS2 group, the mortality with a dose of 2.3 x 10^8 CFU/mL of *M. marinus* (IRTA-19-132) was higher, increasing gradually from 1/20 (5%) at 48 hpi, until a maximum of 5/20 (25%) at 7 dpi. There was no significant difference among the mortalities registered in the two experimental groups (Figure 3a). There was no *V. aestuarianus* detected by qPCR nor isolated on media from the MS1 group (injected with *M. marinus* IRTA-19-131). While it was detected in 3/4 of tissue DNA from moribund oysters and isolated on marine agar from 4/4 samples from MS2 group (Injected with *M. marinus* IRTA-19-132).

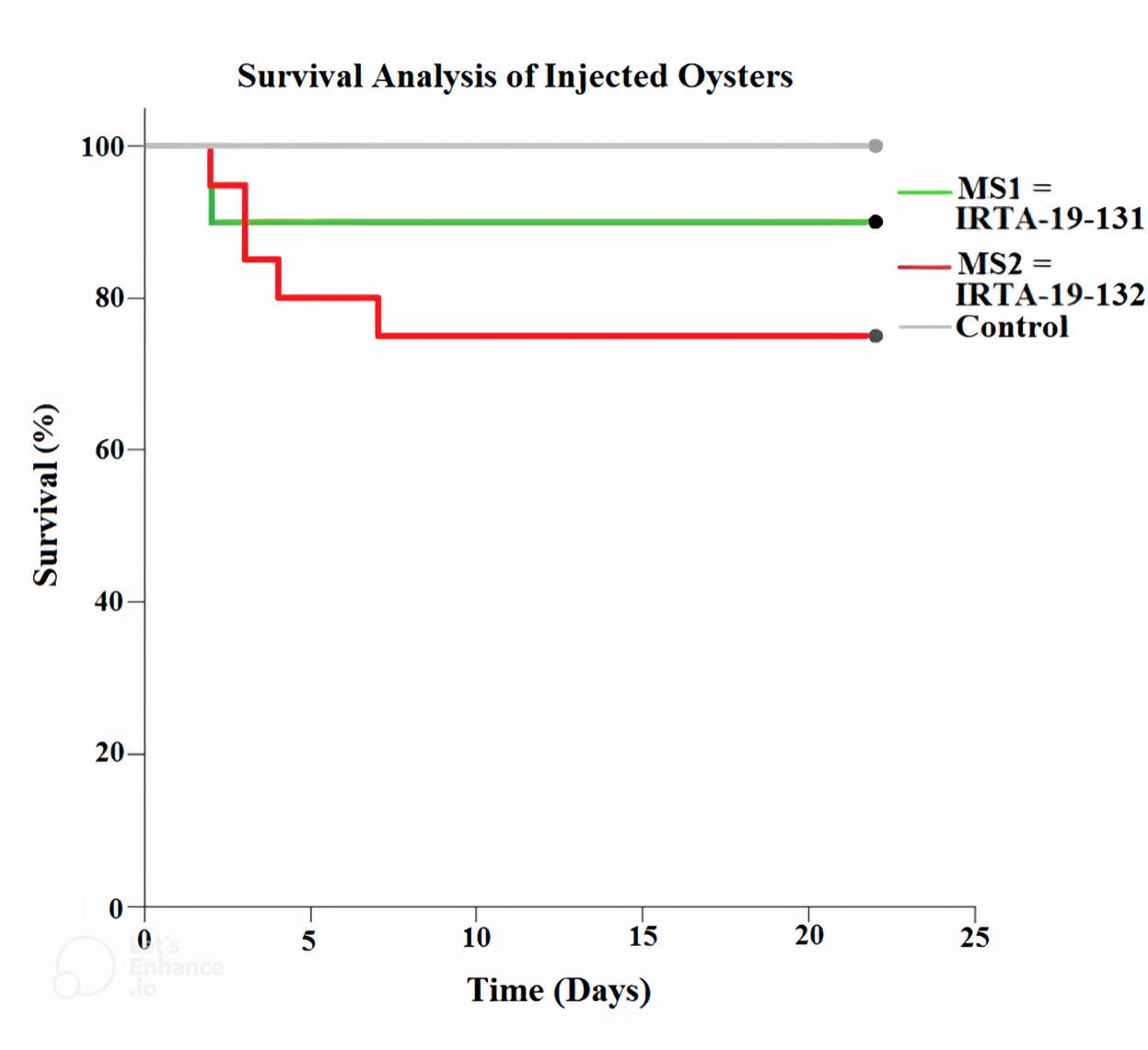


Figure 3a. Kaplan-Maier survival analysis curves of adult oysters challenged by injecting independently into the adductor muscle two strains of *Malaciobacter marinus* at the concentration of 1.8 x 10^8 CFU/mL (MS1, IRTA-19-131, Green line) and 2.3 x 10^8 CFU/mL (MS2, IRTA-19-132, Red line). The grey line represents the control oyster group injected with the same volume of 50% SASW (sterilized artificial seawater).

Experiment 2: Bacterial challenge by cohabitation

The cohabitation experiment would represent the actual scenario taking place in the bay when a disease outbreak occurs. The cohabitation groups were formed after 24 hours in contact (same tank) with two MS2 moribund oysters from experiment 1 and 10 adult fresh oysters. In the fasted oyster group (S2C group) the first mortality (1/10) was observed at 24 hours post cohabitation and reached 50% (5/10) over 12 days (Figure 3b). In the oyster group that was fed with phytoplankton (S2D), the first mortality was observed at day five post-cohabitation (40% of mortality) and continued until day 14 post cohabitation, with a cumulative mortality of 90% (9/10). It was found that with cohabitation a significant increase in the mortality ($p < 0.05$) was observed compared to the injection experiment There was 40% lower survival in the oyster group feed with phytoplankton during cohabitation with IRTA-19-132 than in the fasted oyster group, but there was no statistically significant difference ($p= 0.70$) between the two experiments, i.e., fed vs non-fed.

There was no detection by qPCR of *V. aestuarianus*, *V. splendidus*-clade and *Arcobacter*-like spp. in the phytoplankton mixture assays and no growth with conventional microbiological culturing.

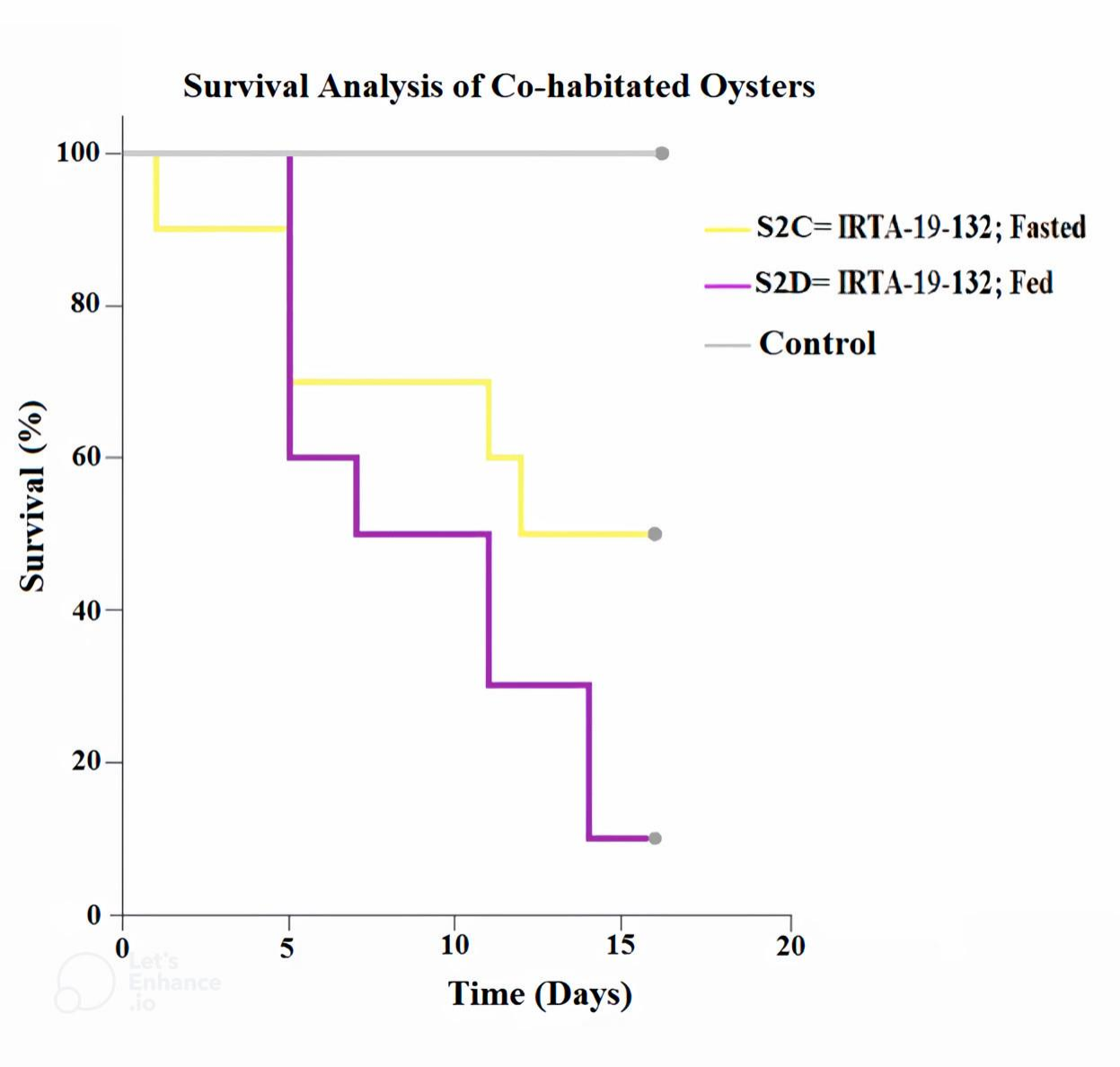


Figure 3b. Kaplan-Maier survival analysis curves of oysters challenged by cohabitation with infected oyster after injecting them with *Malaciobacter marinus* strain IRTA-19-132. The yellow line represents the survival of the fasted oysters (S2C group), and the purple line represents the survival of oysters fed with phytoplankton (S2D group). The grey line represents the control oyster group not exposed to infected oysters.

There was no detection by qPCR of either OsHV-1 nor *V. splendidus* in the tissue samples of all moribund oysters, from both injection (experiment1) and cohabitation challenge (experiment 2) (Supplementary table 4). Moreover, *V. splendidus* clade bacteria were not isolated from any sample (Supplementary table 4). The moribund oysters from the fasted co-habitation group, *M. marinus* was detected and isolated from all moribund oysters (100%), while *V. aestuarianus* was detected in 3/4 oyster tissue DNA. In the fed cohabitation oyster group (S2D), *M. marinus* was detected in 4/8 oyster tissue DNA, while it was isolated from all moribund oysters. In all moribund oysters *V. aestuarianus* was isolated but was only detected in tissue DNA of 5/8 oysters. The injected strains of *M. marinus* were re-isolated from moribund oyster tissues (Supplementary table 4).

The histological examination of the moribund oyster injected with *M. marinus* strain IRTA-19-131 of experiment 1 shows tissue lesions into the gonads and digestive glands due to hemocytic infiltration as response of the host against the bacteria, after the challenging the oysters (Figure 4).

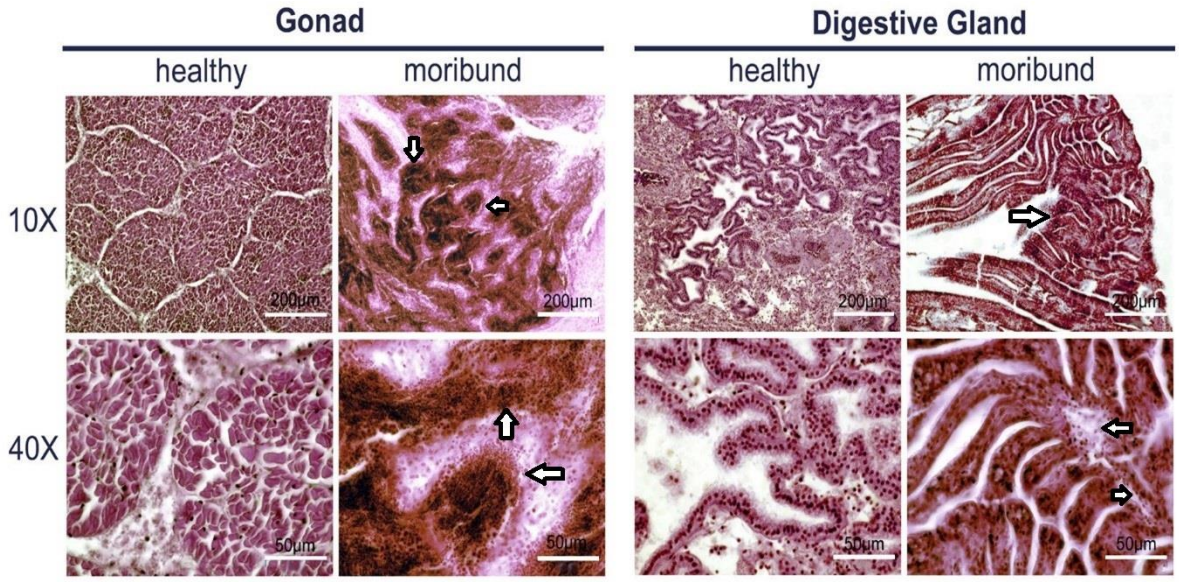


Figure 4: *Crassostrea gigas* representative images of oysters experimentally challenged with *Malaciobacter marinus* strains by injection from experiment 1. Hematoxylin and eosin (H&E) staining of the tissue shows hemocytic infiltration, indicated by arrows in moribund oyster samples in the gonad and digestive glands.

Experiment 3: *V. aestuarianus* challenge experiment

The first mortality of the adult oysters was observed two dpi with *V. aestuarianus* (IRTA-19-106) with two doses i.e., 10^7 CFU/ml (40% mortality) and 10^8 CFU/ml (20% mortality). However, with a dose of 10^6 CFU/ml the first mortality with this strain was observed six dpi (20% mortality). A 100% mortality with this *V. aestuarianus* strain IRTA-19-106 was observed within five dpi with both the 10^7 CFU/ml and 10^8 CFU/ml injection dose (Figure 5a), while with the 10^6 CFU/ml dose of the same strain, 80% mortality was observed 10 dpi. No mortality was observed in either control groups.

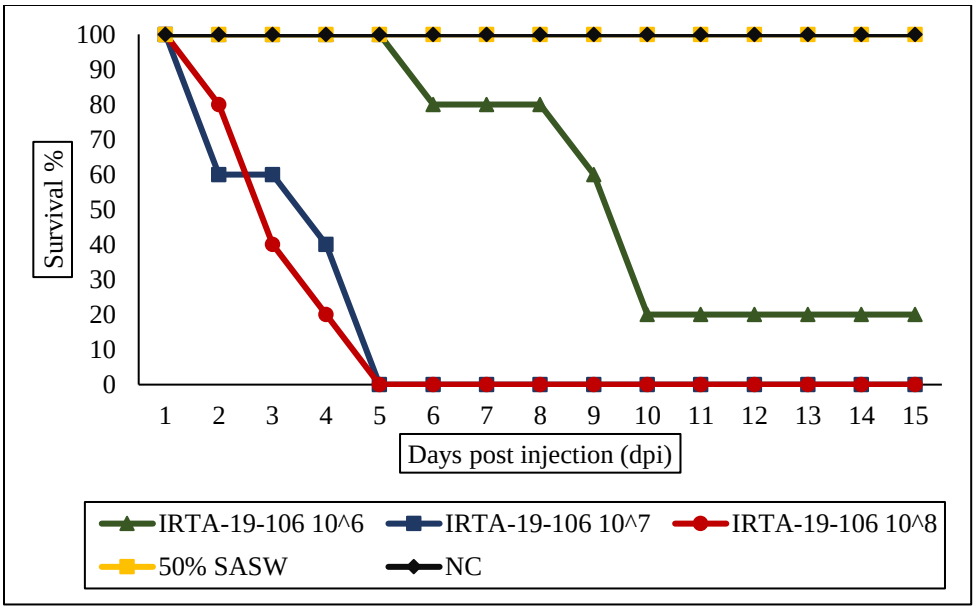


Figure 5a. Survival (%) of adult oysters (*Crassostrea gigas*) challenged by injecting 0.1 ml of 10^8 CFU/ml (Red line), 10^7 CFU/ml (Blue line) and 10^6 CFU/ml (Green line) of *Vibrio aestuarianus* strain IRTA-19-106. The black and yellow line represents the negative control and oysters injected with the same volume of 50% SASW (sterilized artificial seawater) respectively, into the adductor muscle.

In the oysters injected with *V. aestuarianus* IRTA-19-107 the first mortality (40%) was observed in the animals injected with the highest dose of 10^8 CFU/ml after two dpi and increase to 100% mortality six dpi (Figure 5b). While with doses of 10^7 and 10^6 CFU/ml the first mortalities were observed at four dpi (20% mortality) and seven dpi (20% mortality), respectively. A 100% mortality with the dose 10^7 CFU/ml of *V. aestuarianus* strain IRTA-19-107 was observed at 9 dpi. While the highest mortality with the dose 10^6 CFU/ml of the same strain was 60% that was observed at seven dpi and then no more mortality was observed in this group of animals. The 100% mortality observed in the *C. gigas* with *V. aestuarianus* strain IRTA-19-106 was

comparatively quicker than the one generated with strain IRTA-19-107. It was observed that 100% mortality occurred after five dpi of *V. aestuarianus* strain IRTA-19-106 in both 10^7 CFU/ml and 10^8 CFU/ml doses while the same mortality of 100%, with *V. aestuarianus* strain IRTA-19-107, occurred after six dpi at a dose 10^8 CFU/ml and nine dpi at a dose of 10^7 CFU/ml.

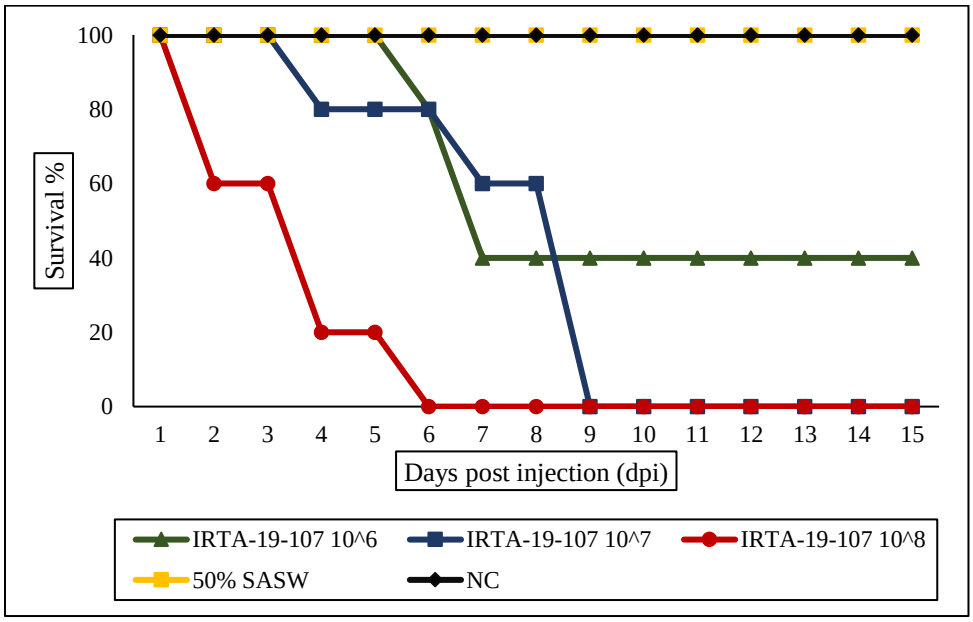


Figure 5b. Survival (%) of adult oysters (*Crassostrea gigas*) challenged by injecting 0.1 ml of 10^8 CFU/ml (Red line), 10^7 CFU/ml (Blue line) and 10^6 CFU/ml (Green line) of *Vibrio aestuarianus* strain IRTA-19-107. The black and yellow line represents the negative control and oysters injected with the same volume of 50% SASW (sterilized artificial seawater) respectively, into the adductor muscle.

In the moribund oysters, *V. aestuarianus* was detected by screening of oyster tissue DNA by duplex TaqMan qPCR and isolated on marine agar media (data no showed). The Ct (cycle

threshold) values were between 12 and 16 from the tissue DNA of these moribund oyster while the CFU/ml range was 3.7 x 10^6 to 2.4 x 10^8 CFU/ml. No other pathogen like OsHV-1, *V. splendidus* clade, *Arcobacter*-like spp., or *M. marinus* were detected or isolated from these moribund oysters of the *V. aestuarianus* challenge experiment.

Experiment 4: Dual challenge of oysters with *M. marinus* and *V. aestuarianus* strains

There was no mortality observed in both the control i.e., NC (anesthesia control) and SASW (injection control with 0.1ml of 50% sterilized artificial seawater). There was no mortality this time in the oysters injected with only *M. marinus* strains IRTA-19-131 (group MS1) and IRTA-19-132 (group MS2) at a dose of 10^8 CFU/ml. The first mortality was observed at two dpi where 5% and 10% of the oysters died in group MV316 (injected with *M. marinus* IRTA-19-131 dose 10^8 CFU/ml + *V. aestuarianus* IRTA-19-106 dose 10^7 CFU/ml) and group MV326 (injected with *M. marinus* IRTA-19-132 dose 10^8 CFU/ml + *V. aestuarianus* IRTA-19-106 dose 10^7 CFU/ml), respectively (Figure 6).

Mortalities started on day three post infection, for all groups challenged with *V. aestuarianus*, in contrast with those with *M. marinus* only, which registered no mortalities. However, the survival / mortality pattern in the oysters challenged only with VA6 [IRTA-19-106] (dose 10^7 CFU/ml) was gradual, stepwise for nine days, like in previous experiments (experiment 3) of VA6 [IRTA-19-106] (dose 10^6 CFU/ml), with similar % of survivors (25%). Dual challenged animals presented a more acute mortality picture, between day three and six with overall mortalities higher than in the single VA6 [IRTA-19-106] challenge group.

There was no significant ($p<0.05$) difference among the dually challenged oyster groups i.e., MV316 and MV326. Although the mortality was comparatively higher but not significant

(*p>0.05*), within the dually challenged oyster groups i.e., MV316 and MV326, than the oyster group singly challenged with *V. aestuarianus* IRTA-19-106. There was no detection of OsHV-1 and *V. splendidus* clade nor isolation from the moribund oyster tissue screened by qPCR assays or bacterial culturing (data not shown). The Ct value for *V. aestuarianus* ranged from 14 to 18 and the colony count on Marine agar were between 6.2 x 10^6 CFU/ml to 4.1 x 10^7 CFU/ml while for *M. marinus* the Ct values ranged from 23 to 27 and the colony count was 4.7 x 10^6 to 5.9 x 10^6.

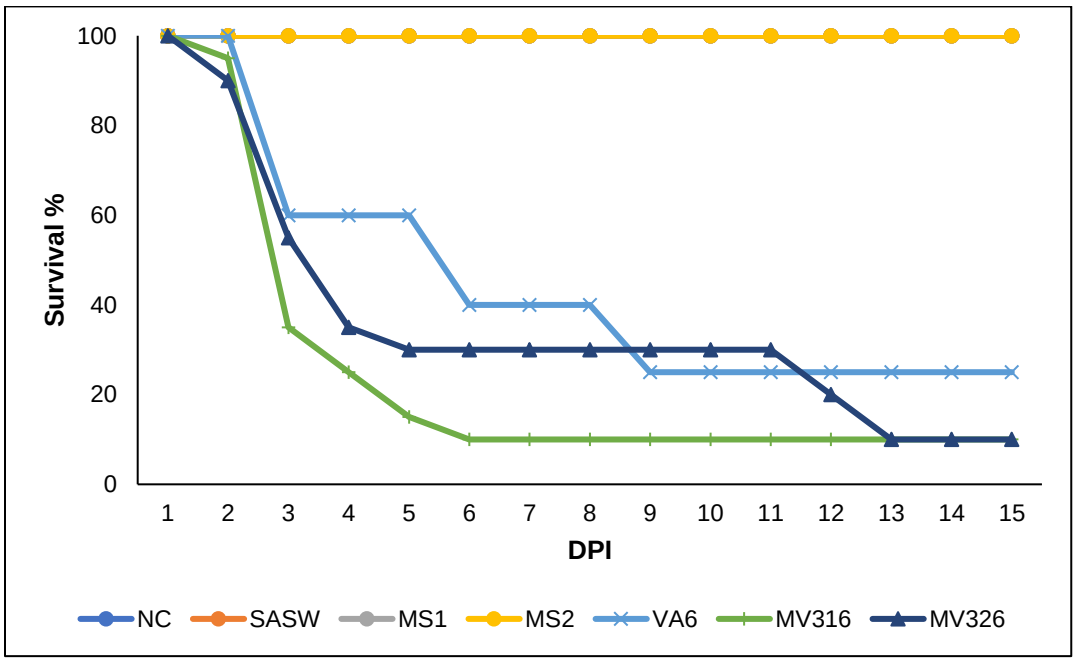


Figure 6: Survival curves of Pacific oyster (*Crassostrea gigas*) during the dual challenge experiment with bacterial strains of *M. marinus* (IRTA-19-131 & IRTA-19-132 with doses of 10^8 CFU/ml each) and *V. aestuarianus* (IRTA-19-106 dose 10^7 CFU/ml). NC= Anesthesia control. SASW= injection control, injected with 50% artificial seawater. MS1= Injected with *M. marinus* IRTA-19-131. MS2= Injected with *M. marinus* IRTA-19-132. VA6= Injected with *V.*

aestuarianus IRTA-19-106. MV316= Dually challenged with *M. marinus* IRTA-19-131 and *V. aestuarianus* IRTA-19-106. MV326= Dually challenged with *M. marinus* IRTA-19-132 and *V. aestuarianus* IRTA-19-106.

In vitro experiments with *C. gigas* hemocytes

In vitro effect of *M. marinus* challenge on hemocytes lysosomal membrane stability

The effect of *M. marinus* IRTA-19-131 challenge on *C. gigas* hemocytes lysosomal membrane stability (LMS) was assessed by neutral red retention time assay (NRRT assay) and the results are given below (Figure 7). The results show that in the higher *M. marinus* IRTA-19-131 concentrations of 10^8 and 10^7 CFU/ml the lysosomes were very unstable compared to the control (-96.9%, and -90.8% respectively; $p < 0.01$). At the lower bacterial concentration of 10^6 CFU/ml the stability of the lysosome hemocytes increased compared to the previous two higher bacterial concentration, but still lower compared to the control (-43.1%, $p < 0.05$). These results indicate that the hemocytes lysosomal membrane stability is dependent on *M. marinus* IRTA-19-131 dose concentration. With higher doses like 10^8 and 10^7 CFU/ml these bacteria can destabilize the lysosomal membrane very fast and, in less time i.e., as soon as exposed to the higher doses. This effect on the lysosome was highly significant ($p < 0.01$) in the presence of higher bacterial doses. While in the case of lower doses of 10^6 CFU/ml there was not a rapid decrease in the stability of the lysosomes, but still the affect was significant ($p < 0.05$).

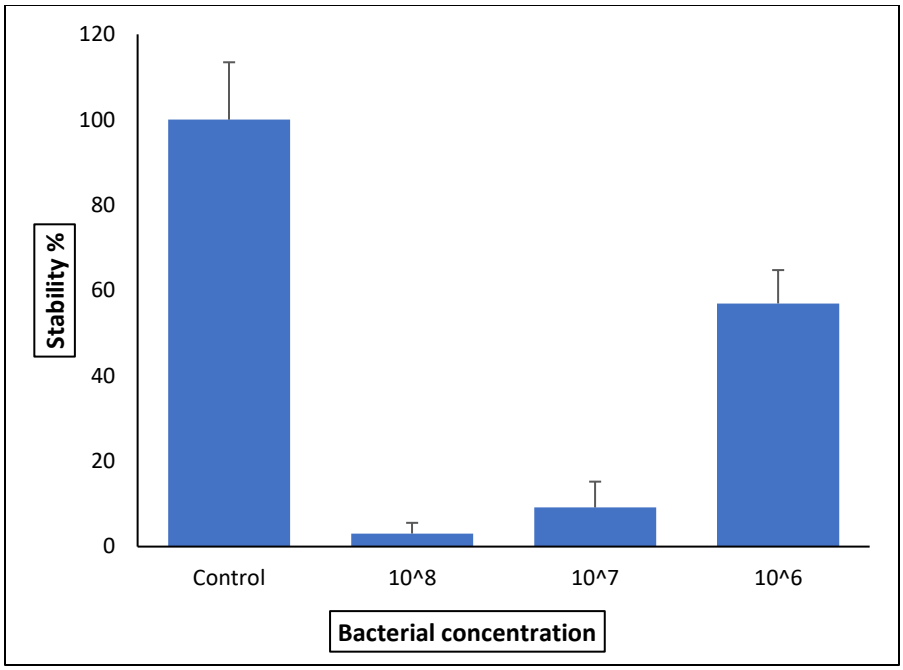


Figure 7. Lysosomal membrane stability of *Crassostrea gigas* hemocytes assessed by neutral red retention time assay challenged *in vitro* with three concentrations (10^8, 10^7 and 10^6 CFU/ml) of *Malaciobacter marinus* strain IRTA-19-131 isolated in June-2019 summer mortality of *C. gigas* in Ebro Delta, Spain. In the control hemocytes sterilized artificial seawater were used instead of bacterial suspension to correlate with the samples treated with bacteria. The data (arithmetic mean ± SD) are expressed as percentage of the values as observed to normalize the values in terms of percentage and is the result of five independent experiments carried out in triplicates.

Bactericidal killing assay

In the bactericidal killing assay, after 30 minutes of incubation of hemocytes with *M. marinus* a 39.5% killing was observed (Figure 8) compared to control ($p < 0.05$) which shows the initial killing of the bacteria by *C. gigas* hemocytes in the initial phase of the infection. Later, after

incubation of 90 minutes the killing was reduced to only 26% ($p < 0.05$) compared to the T0 control which is an increase of 13.5% in growth compared to killing after 30 minutes incubation.

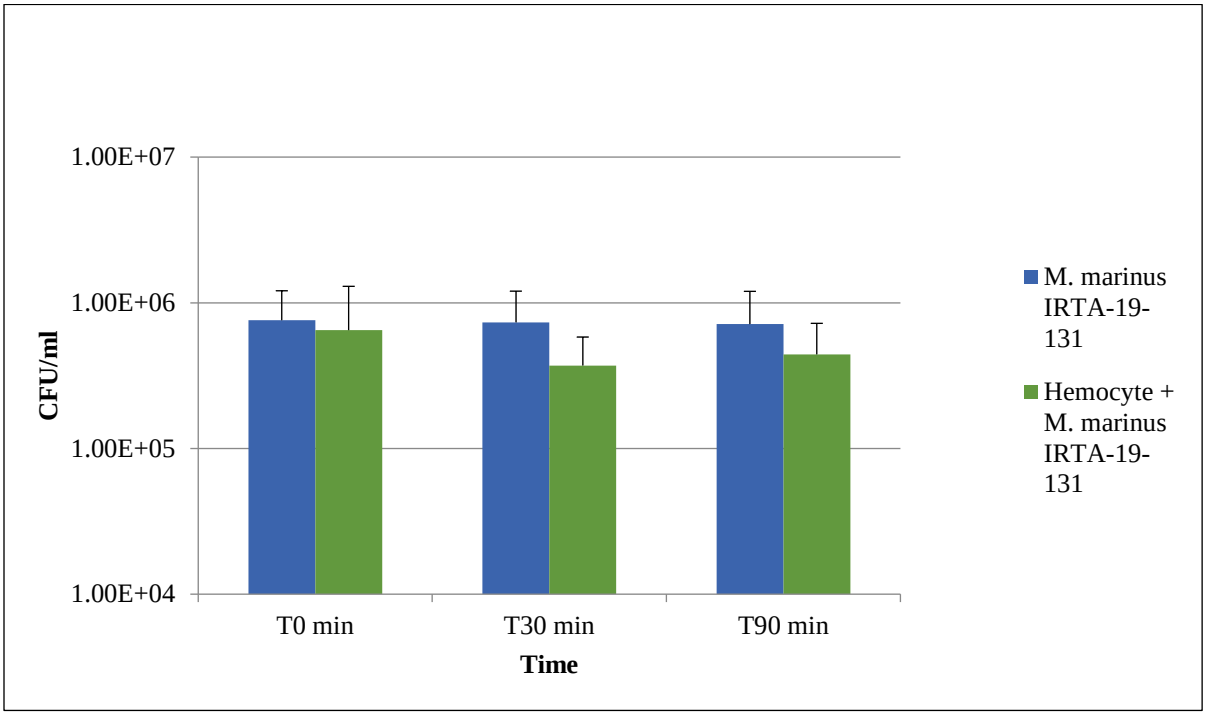


Figure 8. *In vitro* bactericidal activity of *Crassostrea gigas* hemocytes incubated with 1 x 10^6 CFU/ml of *Malaciobacter marinus* IRTA-19-131 for 0, 30 and 90 minutes. As a control, the bacterial inoculum without hemocytes were incubated in the same condition and time. The results are expressed as number of cultivable bacteria CFU/hemocyte monolayer.

Discussion

Arcobacter-like spp. have been found from terrestrial and marine origins and among them are species that can be pathogenic for humans, but to our knowledge no study has reported their pathogenicity to marine animals and specifically for Pacific oysters except for *Mytilus*

galloprovincialis larvae (Auguste et al., 2022). Recently, the species *Arcobacter mytili* of marine origin recovered from mussels (Collado et al., 2009b) now known as *Malaciobacter mytili* (Perez-Cataluña et al 2018), was reported, for the first time, to cause bacteremia in a man working in a crab farm, suggesting the pathogenic nature of this group of bacteria within *Arcobacter*-like species (Vasiljevic et al., 2019). The rise in isolation of *Arcobacter*-like spp. from the marine environment and specifically from shellfish shows that this group of bacteria may have some important role in the marine environment, and specifically in shellfish like Pacific oysters. In fact, our previous studies showed that recovery of marine *Arcobacter*-like spp. was significantly enhanced by the addition of 2.5% NaCl (w/v) to the Arcobacter-CAT enrichment broth followed by culturing on marine agar (Salas-Massó et al., 2016), or by adding 50% seawater (Rahman et al., 2020). In this way, Salas-Massó et al. (2016) recovered ca. 40% more positive samples and in addition recovered 11 known species and seven species new to science some of them still pending to be described (Perez-Cataluña et al 2018). Among the former they found for the first records since their original description of the species *A. marinus* (Kim et al., 2010) and *A. halophilus* (Donachie et al., 2005). Altogether, indicating the ubiquity of the genus in the marine ecosystems.

These species are now known to belong to the genus *Malaciobacter* (Peréz-Cataluña et al., 2016). When we compared the recovery potential from field samples of the Arcobacter broth supplemented with 50% ASW (Artificial seawater), with that of 2.5% NaCl (Salas-Massó et al., 2016), *Arcobacter*-like spp., were isolated in 100% of the oysters, of the June-2019 mortality episode using the former method, while with the latter method only 50% of field samples were found positive. This shows that *Arcobacter*-like spp. in marine environments, and specifically in

Pacific oyster require specific salts and ions other than NaCl for their growth and isolation on culture media, and this is provided by ASW.

Genotyping might play an important role in epidemiological surveillance. Several genotyping techniques have been used to differentiate *Arcobacter*-like strains and trace their transmission route and source of infection in animals and humans (Ramees et al., 2017). The different genotyping techniques used for *Arcobacter*-like isolates included ERIC-PCR, RAPD-PCR, AFLP and PFGE (Houf et al., 2002b; On et al., 2002; Ongor et al., 2014). Specifically, ERIC-PCR and RAPD-PCR have been used for genotyping *A. butzleri*, *A. skirrowii* and *A. cryaerophilus* and both have reasonable discriminatory power, but the ERIC-PCR patterns have been shown to be more reproducible than RAPD-PCR pattern in some studies (Houf et al., 2002b). In this study, ERIC-PCR was applied to *Arcobacter*-like isolates recovered from oysters to reveal the genetic diversity in our isolates and to find the abundance of each genotype in our samples (Table 2). Based upon this primary screening by ERIC-PCR, it was found that the genetic diversity of *Arcobacter*-like isolates was higher in the isolates recovered with the Arcobacter broth + 50% ASW (Nine different ERIC-PCR genotypes) compared to Arcobacter broth + 2.5% NaCl (Two genotypes). Further analysis of those nine genotypes showed that there were seven new genotypes isolated with Arcobacter broth + 50% ASW, while the two genotypes isolated using 2.5% NaCl enrichment were also isolated with the Arcobacter broth + 50% ASW broth medium. Similar results were also observed by Salas-Massó (2016) where 8.6% more new genotypes were isolated from shellfish with the Arcobacter CAT broth +2.5% NaCl than with the Arcobacter CAT enrichment broth alone.

One isolate from each of the different ERIC-PCR genotype was selected, to avoid working with "sister" clones, for their identification using the phylogenetic analysis of the *rpoB* gene

sequences. The *rpoB* nucleotide BLAST analysis of consensus DNA sequences from the isolates recovered from the mortality event showed that 78% of the *Arcobacter*-like isolates were strains of *M. marinus* (99.6%-100% similarity). This is the first time, to our knowledge, that this species has been isolated from moribund adult oysters during a mortality event. Therefore, this species was suspected to have a possible role in causing the mortality in adult Pacific oysters. Moreover, a potential novel isolate IRTA-19-136 clustered with candidate species "*Malaciobacter neptunis"* (F146-38^T = CECT 8986^T = LMG 29054^T) isolated previously from mussels from Alfacs Bay, Ebro River Delta (Peréz-Cataluña et al., 2018). The sequencing of two further housekeeping genes *atpA* and *gyrB* gene for the IRTA-19-132, IRTA-19-133 and IRTA-19-136 followed by making a concatenated sequence (1602bp) of *atpA*, *gyrB* and *rpoB* genes as mentioned by Levican et al. (2013b) and then construction Neighbor-Joining tree resulted in the confirmation that IRTA-19-132 belongs to *M. marinus*. While the other two IRTA-19-133 and IRTA-19-136 are potentially novel *Arcobacter*-like spp. which should be characterized to properly describe them as novel *Arcobacter*-like spp.

The virulence potential of marine *Arcobacter*-like spp. is not known yet. It has been reported that LPS of *M. halophilus* may play a role in the survival of this species in marine environment (Silipo et al., 2010). It has been found that *Arcobacter*-like spp. show strain-specific differences in invasion and survival capacity inside the human intestinal cells (Levican et al., 2013a) and human macrophages (Zur Bruegge et al., 2014). *Arcobacter*-like spp. are motile, and this may also have a role in invading and colonizing as reported in other pathogenic bacteria (Ho et al., 2008b). Ten putative virulence genes have been studied in *Arcobacter*-like spp. and it has been found that *A. butzleri* LMG10828^T possessed at least ten putative virulence genes (*ciaB, hecA, hecB, cj1349, cadF, mviN, pldA, tlyA, irgA* and *iroE*), (Douidah et al., 2012; Levican et al.,

2013a; Ferreira et al., 2014b). These putative virulence genes have different functions: *ciaB* helps in invasion of host cell, *hecA* is a member of filamentous hemagglutinin family, while the hemolysin activation protein is encoded by *hecB*. Both the *cj1349* and *cadF* genes encode for outer membrane proteins that help in facilitating cell to cell contact to epithelial cells of the intestine by adhering to fibronectin. The *mviN* gene is involved in peptidoglycan and cell membrane synthesis, and *pldA* encodes phospholipase A in the outer membrane of bacteria that hydrolyzes acyl ester bonds. More significant is *tlyA* that encodes hemolysin, which can be directly implicated in host cell damage. The gene *irgA* encodes for an outer membrane receptor for enterobactin, a siderophore and potential source for bioavailable iron, and *iroE* helps in iron acquisition and maintaining infection and as with many pathogenic species, iron can be limiting for growth within a host (Douidah et al., 2012; Ramees et al., 2017). A recent study has also found these ten virulence genes in the genomes of two *A. butzleri* strains isolated from the marine clam, *Tapes philippinarum* and the mussel, *Mytillus galloprovincialis* purchase from local market in Italy (Fanelli et al., 2019). Most of these putative virulence genes are reported and found in terrestrial *Arcobacter*-like spp. specifically in *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* that are found associated with human and animal diseases (Kim et al., 2019). In our study we found that *M. marinus* strain IRTA-19-132 possessed three known virulence genes (*ciaB*, *cj1349* and *tlyA*), although most of the other *M. marinus* strains isolated in this study possessed none of the previously described virulence genes. These genes found in our strains are responsible for host cell invasion, facilitating cell to cell contact and adherence to epithelial cells, and production of hemolysin. The negative results for many of these virulence factors in *M. marinus* could be due to several reasons. For instance, the genes are not present, the sequence of primers are based on different species. Because of this it could be interesting to search for new

virulence factors or confirm their presence in the genome of the bacteria. The expression of virulence and pathogenesis by bacteria is influenced by temperature and salinity in the environment (Vezzulli et al., 2013), which could be the reason that *Arcobacter*-like spp. were isolated in the summer mortality in this study. Other studies reported that the abundance of *Arcobacter*-like spp. increased when the water temperature increased (Lokmer and Wegner 2015; King et al., 2019). A study in mussels (*M. galloprovincialis*) showed that with the increase of temperature, *Arcobacter*-like spp. proliferated, concluding that temperature plays a key role in causing a shift in the gut microbial communities of these mussels (Li et al., 2019). The augment of opportunistic pathogens also boosts the susceptibility of the host to diseases and contributes to mass mortalities in *M. galloprovincialis* (Li et al., 2019). Many new *Arcobacter*-like strains and species isolated from oyster are reported in the present study. Although *Arcobacter*-like spp. have not yet been specifically associated with disease in shellfish, several microbiome-based studies have found that growth of *Arcobacter*-like spp. increased in *C. gigas* during mortality events attributed to *Vibrio aestuarianus* and/or OsHV-1 (Lasa et al., 2019). Lokmer and Wegner (2015) found that hemolymph of moribund oysters is dominated by members of the genus *Arcobacter* and suggested that they are opportunistic pathogens.

In experiment 1, where two *M. marinus* isolates, IRTA-19-131 and IRTA-19-132, were injected into adult *C .gigas,* mortalities of 10% and 25%, respectively followed, and, although there was no statistically significant differences, strain IRTA-19-132, harboring 3 virulence genes (*ciaB, cj1349* and *tlyA*), did have higher mortalities suggesting these virulent genes may have a role in pathogenicity of *M. marinus* in adult Pacific oysters. The putative virulence of strain *M. marinus* IRTA-19-132, was more apparent during experiment 2, where a challenge by co-habitation was carried out with a moribund oyster from experiment 1. This cohabitation challenge experiment

mimics the actual conditions faced by *C. gigas* in marine environments where they actively filter feed and accumulate the pathogens inside their gut (Richard et al., 2021). The histological examination shows the hemocytic infiltration response of the host oyster challenged with *M. marinus* in both the injection model and the cohabitation model in the gonad and digestive glands of the oysters. Hemocytic infiltration is an immune response of the host oysters against the foreign invading particles or pathogens (Allam and Espinosa, 2016; Gerdol et al., 2018). Similar effects have been observed in several studies where hemocytic infiltration has been observed in the oysters (Gomez-Leon et al., 2008; Mandas et al., 2020). The findings in this study highlight the importance of further studies of *Arcobacter*-like spp. to properly elucidate their role in causing the mortalities in shellfish, specifically *C. gigas.*

In the screening of baseline samples, we encountered *V. aestuarianus,* a known pathogen for Pacific oyster (Garnier et al., 2007; Solomieu et al., 2015; Pernet et al., 2016; Azema et al., 2017; Travers et al., 2017; Petton et al., 2019), and during the challenge experiment in the MS2 group (injected with IRTA-19-132). These depurated oysters were bought for the experiment from the depuration plant and there was no mortality in any of the control groups i.e., NNC, AC, SWC, nor was there detection of it in the MS1 group (injected with IRTA-19-131) where the mortality observed was attributed to the injection of the *M. marinus* strain. Furthermore, it highlights the importance of using SPF (specific pathogens free) oysters for such kind of experiments to avoid unwanted contamination during the experiments. The finding of both bacteria simultaneously suggests their possible role in causing the mortality of Pacific oysters under these experimental conditions. Both *V. aestuarianus* strains IRTA-19-106 and IRTA-19-107, isolated from the same mortality episode, behave very similarly, in terms of kinetics of the mortality/ survival curves and act in a dose- response manner providing a dose range to establish future lethal dose

experiments. The genome analysis of both strains has shown that both these strains are clones (Mensil et al., 2022).

In the dual challenge it was observed that the onset of the mortality occurred quickly i.e., at two dpi, but also the intensity of the mortality was higher than the singly challenged oyster with only the *V. aestuarianus* strain. The intensity of the mortality in the dual challenged oysters was so high that after three dpi almost 50% mortality was observed in both groups. While in the oysters injected with only the *V. aestuarianus* strain, 50% mortality was observed later at six dpi. Further, the final survival percentage was also lower in the dually challenged than singly challenged *V. aestuarianus* oyster group i.e., 10% vs 25%. These bacteria (*V. aestuarianus* and *Arcobacter*-like spp.) have also been reported in hemolymph and in the microbiome of Pacific oysters (Lokmer and Wegner 2015; Lasa et al., 2019; Richard et al., 2021). In addition, *M. marinus* has been associated with the dysbiosis of *C. gigas* normal microbiota that occurs post OsHV-1 viral infection (Petton et al., 2021). These findings suggest that the combined infection of *V. aestuarianus* and *M. marinus* might represent a larger threat to *C. gigas* aquaculture sector.

Regarding the *in vitro* experiments performed with hemocytes, a similar affect was observed with hemocytes of other bivalves such as *M. galloprovincialis* where *Vibrio splendidus* LGP32, isolated from oyster mortality episode, induced 82% decrease in stability of the lysosome while *V. aestuarianus* 01/032 (Labreuche et al., 2006a; Pezzati et al., 2015) did not induce this stress (Balbi et al., 2013). This may suggest that *C. gigas*, being a filter feeder, when present in an environment where the animal accumulates *M. marinus* in high quantity, it may give a stress to the animal. This condition could be fulfilled in a mortality episode where the infected or dying oysters may shed the bacteria to the surrounding environment and accumulated by nearby animals on the same rope or in other nearby environments. As hemocytes have a protective role

against bacterial, viral, and chemical contaminants in bivalves and plays role in the homeostasis of the animals, this stress and destabilization of the hemocytes may lead to infection by other opportunistic bacterial and viral pathogens such as several species of *V. splendidus* clade or other pathogens like OsHV-1 and *V. aestuarianus* etc. thereby increasing the severity of the mortality episode. Other bacteria such as *V. aestuarianus* 01/032, a strain originally isolated from a mortality, was also found pathogenic to juvenile *C. gigas* (Garnier et al., 2007) and was able to give stress to the hemocytes and secrete an extracellular metalloprotease to impair functional responses of these cells (Labreuche et al., 2006b; 2010). The killing ability of *C. gigas* hemocytes was evaluated by bactericidal killing assay by incubating the *M. marinus* IRTA-19-131 with 10^6 CFU/ml concentration at different incubation time. The assay quantifies the number of cultivable, live bacteria after incubation with hemocytes (Balbi et al., 2018; Auguste et al., 2022). The results indicate that although during initial infection of *C. gigas* hemocytes by *M. marinus*, in an *in vitro* experiment, the bacteria is neutralized and killed by the hemocytes, but this bacterium can resist to hemocyte killing and start growth again in presence of the *C. gigas* hemocytes. The decrease in bacterial killing with more incubation time could be associated with some product produced by the bacteria that could cause morphological change and immune suppression in *C. gigas* hemocytes as reported in the case of *V. aestuarianus* that caused this affect in *C. gigas* hemocytes during *in vitro* study (Labreuche et al., 2006a). Another study also reported that *C. gigas* hemocyte did not kill *V. aestuarianus* isolated from a mortality episode (Pezzati et al., 2015). But no such study has been done with *M. marinus* before with which a similar effect can be compared.

This is the first study where the interaction of *M. marinus* individually and in a combined challenge with the known *C. gigas* pathogen i.e., *V. aestuarianus* strains, both isolated during an

outbreak from Ebro Delta, Spain. Furthermore, it is the first study where *M. marinus* has been isolated concurrently with *V. aestuarianus* from the same mortality episode and studied using both *in vivo* and *in vitro* challenge experiments with *C. gigas.* The preliminary results obtained during the present study show a comparatively severe effect of both *M. marinus* and *V. aestuarianus* on *C. gigas* in inducing mortality. Therefore, keeping in view the results from the present study, further studies should be continued on the disease interaction and relationship of these two important bacteria in shellfish, specifically *C.* gigas.

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Authors contributions

F.U.R. performed experiments, interpreted the experimental data, and drafted the manuscript. F.U.R., K.B.A. and M.D.F. designed the experiments. F.U.R and M.F.T performed the statistical analysis. M.D.F., K.B.A., M.F.T. and M.J.F supervised the study. All authors contributed and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Correspondence and requests for materials should be addressed to the M.D.F.

Supplementary Materials

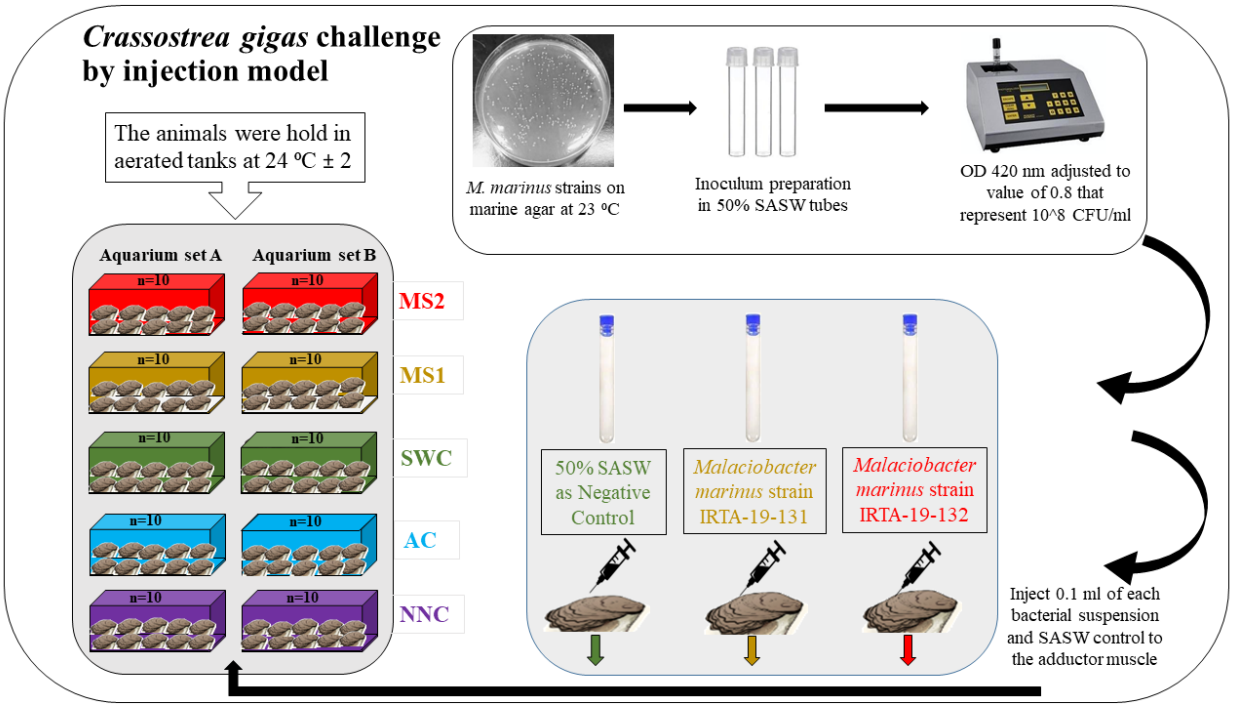
Supplementary table 1: Primer's name, sequences and amplicon size used in this study for identification of *Arcobacter*-like spp. and for the detection of putative virulence genes in *Arcobacter*-like isolates from *C. gigas* from Ebro Delta, Spain.

PCR	Primer Pair	Function	Sequence of Primers (5´to 3´)	Amplicon Size (bp)	Source
Arcobacter 23S rDNA	23S-F 23S-R	Structure of LSU ribosome	AACATATAAGCGCGATGTGGGGAC ACGGTACGGGCAACATATAATA	233	Salas-Masso et al., 2019; Hausdorf et al., 2013
ERIC PCR	ERIC-1R ERIC-2	(genotyping)	ATGTAAGCTCCTGGGGATTCAC	Variable number/length of	Houf et al., 2002

			AAGTAAGTGACTGGGGTGAGCG	DNA fragments	
rpoB	rpoB-Arc15F rpoB-Arc24R	DNA-dependent RNA polymerase subunit B	TCTCAATTTATGGAYCAAAC AGTTATATCCATTCCATGGCAT	845	Salas-Masso et al., 2016
gyrB	gyrB-Arc-7F gyrB-Arc-14R	Encode DNA gyrase-subunit B	GTTTAYCAYTTTGAAGGTGG CTAGATTTTTCAACATTTAAAAT	680	Collado et al., 2011
atpA	atpA-Arc5F atpA-Arc12R	Encode ATP synthase α-subunit	GATACAATTCTTAACCAAAAAGG AAAACTTCWACCATTCTTTG	751	Levican, 2013
Mma rpoB	Mma rpoB-F Mma rpoB-R	(Identification of *M. marinus*)	GATTGTAGAGCCTTTAATTGAAG GTTCTAAGAATGGAATAAGTGAG	135	This Study
cadF	*cadF*-F *cadF*-R	Encodes Fibronectin-binding protein	TTACTCCTACACCGTAGT AAACTATGCTAACGCTGGTT	283	Douidah et al., 2012
ciaB	*ciaB*-F *ciaB*-R	Encodes Campylobacter invasion	TGGGCAGATGTGGATAGAGCTTGGA TAGTGCTGGTCGTCCCACATA	284	Douidah et al., 2012

		antigen	AAG		
cj1349	*cj1349*-F *cj1349*-R	Encodes Fibronectin-binding proteins	CCAGAAATCACTGGCTTTTGAG GGGCATAAGTTAGATGAGGTTCC	659	Douidah et al., 2012
irgA	*irgA*-F *irgA*-R	Encodes an iron-regulated outer membrane protein	TGCAGAGGATACTTGGAGCGTAACT GTATAACCCCATTGATGAGGAGCA	437	Douidah et al., 2012
hecA	*hecA*-F *hecA*-R	Encodes a member of the filamentous hemagglutinin (FHA) family	GTGGAAGTACAACGATAGCAGGCTC GTCTGTTTTAGTTGCTCTGCACTC	537	Douidah et al., 2012
hecB	*hecB*-F *hecB*-R	Encode a hemolysin activation protein	CTAAACTCTACAAATCGTGC CTTTTGAGTGTTGACCTC	528	Douidah et al., 2012
mviN	*mviN*-F *mviN*-R	Encodes an essential protein required for the biosynthesis of peptidoglycan	TGCACTTGTTGCAAAACGGTG TGCTGATGGAGCTTTTACGCAAGC	294	Douidah et al., 2012
pldA	*pldA*-F *pldA*-R	Encodes phospholipase	TTGACGAGACAATAAGTGCAGC	293	Douidah et al., 2012

		A	CGTCTTTATCTTTGCTTTCAGGGA		
tlyA	*tlyA*-F *tlyA*-R	Encodes hemolysin	CAAAGTCGAAACAAAGCGACTG TCCACCAGTGCTACTTCCTATA	230	Douidah et al., 2012



Supplementary figure 1. Experimental design of challenge experiment 1. *Crassostrea gigas* infected with two strains of *Malaciobacter marinus* isolated during a mortality event of adult pacific oysters, from Fangar bay in Ebro Delta, Spain. A similar approach was adopted for other injection challenges i.e., experiment 3 & 4, the only difference was the bacteria, strains, dose, and number of animals that are specified in the material and methods of the respective challenge experiment.

Supplementary table 2: Oysters tissue DNA screening for OsHV-1 (Webb et al., 2007), *Vibrio splendidus* clade, *Vibrio aestuarianus*, (Saulnier et al., 2017), *Arcobacter*-like species (Salas-Masso et al., 2019) and *Malaciobacter marinus* (In preparation) prior to start of the challenge experiment. A tissue DNA concentration of 50 ng/µl was used for each assay. Results from baseline oyster samples of the experiments 1 (*M. marinus* injection experiment), 2 (*M. marinus* cohabitation experiment), 3 (*V. aestuarianus* injection experiment. The code MmChBLS represents the *M. marinus* challenge experiments base line samples.

Sample	**OsHV-1 qPCR**	***Vibrio splendidus* clade qPCR**	***Vibrio aestuarianus* qPCR**	***Arcobacter* 23S qPCR**	***Malaciobacter marinus* qPCR**
MmChBLS-1	Negative	Negative	Negative	Positive (Ct=27.81)	Negative
MmChBLS-2	Negative	Negative	Negative	Positive (Ct=27.51)	Negative
MmChBLS-3	Negative	Negative	Negative	Positive (Ct=30.66)	Negative
MmChBLS-4	Negative	Negative	Negative	Negative	Negative
MmChBLS-5	Negative	Negative	Negative	Positive (Ct=29.12)	Negative
MmChBLS-6	Negative	Negative	Negative	Negative	Negative
MmChBLS-7	Negative	Negative	Negative	Negative	Negative
MmChBLS-8	Negative	Negative	Negative	Positive (Ct=31.58)	Negative
MmChBLS-9	Negative	Negative	Negative	Positive (Ct=26.86)	Negative
MmChBLS-10	Negative	Negative	Negative	Negative	Negative

MmChBLS-11	Negative	Negative	Negative	Positive (Ct=25.42)	Negative
MmChBLS-12	Negative	Negative	Negative	Positive (Ct=31.51)	Negative
MmChBLS-13	Negative	Negative	Negative	Positive (Ct=29.83)	Negative
MmChBLS-14	Negative	Negative	Negative	Negative	Negative
MmChBLS-15	Negative	Negative	Negative	Negative	Negative

Supplementary table 3: Background abundance of *Vibrio aestuarianus*, *Vibrio splendidus* clade, *Arcobacter*-like species and *Malaciobacter marinus* in oysters (*C. gigas*) screened before challenge experiment number 1, 2 & 3. The purified colonies DNA were screened by Duplex vibrio qPCR assay for *Vibrio splendidus* clade and *V. aestuarianus*. While *Arcobacter*-like spp. and *M. marinus* isolates were identified by Arcobacter 23S qPCR and Mma-rpoB qPCR assays.

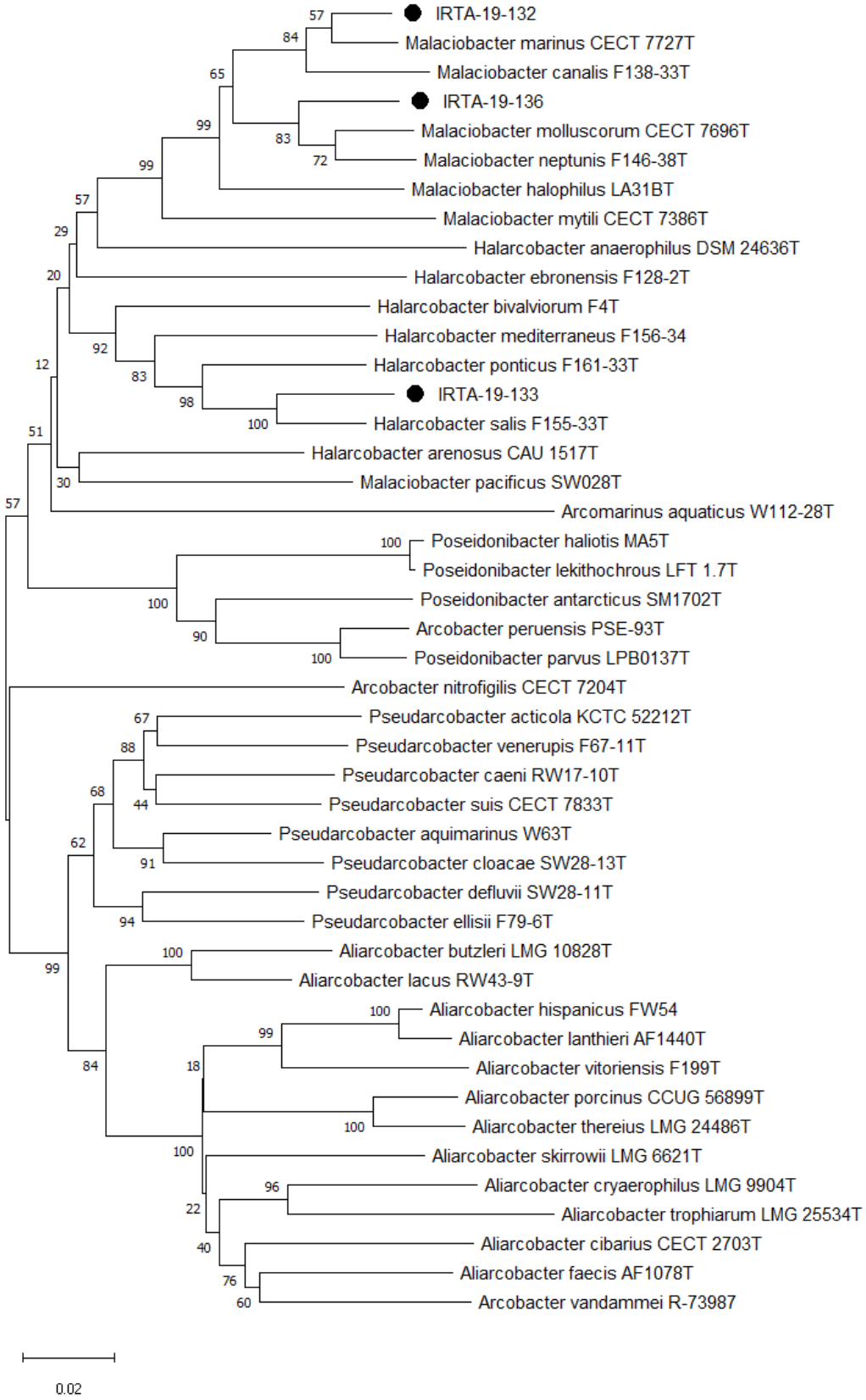
Sample Pool	***Vibrio splendidus* clade grown on TCBS (48h, 23 ºC)**	***Vibrio aestuarianus* grown on Marine agar (48h, 23 ºC)**	***Arcobacter*-like species after enrichment (48h, 23 ºC) followed by isolation on Marine agar (48h, 23 ºC)**	***Malaciobacter marinus* after enrichment 48h, 23ºC) Followed by isolation on Marine agar (48h, 23ºC)**
BPL-1	Negative	Negative	>300, confluent	Negative
BPL-2	Negative	Negative	>300, confluent	Negative
BPL-3	Negative	Positive: 1.2 x	>300, confluent	Negative

		10^4 CFU's/mL		

Supplementary table 4: DNA screening of tissue from moribund oysters, testing for OsHV-1 (Webb et al., 2007), *Vibrio splendidus* clade, *Vibrio aestuarianus* (Saulnier et al., 2017), *Arcobacter*-like species (Salas-Masso et al., 2019) and *Malaciobacter marinus* (In preparation). A tissue DNA concentration of 50 ng/µl was used for each assay. OsHV-1 and *V. splendidus* clade was not detected by qPCR assays while *V. splendidus* clade was also not found in the recovered bacterial isolates hence not shown due to negative results in the table. S1B = *M. marinus* strains IRTA-19-131 tank B; S2A = *M. marinus* strains IRTA-19-132 tank A; S2B = *M. marinus* strains IRTA-19-132 tank B; S2C = *M. marinus* strains IRTA-19-132 cohabitation fasted animals; S2D = *M. marinus* strains IRTA-19-132 cohabitation fed animals. The CFU value refers to the colony count of the positive qPCR results of the genomic DNA isolated from the suspected bacteria based on morphology and calculated according to number of isolates from that specific morphology.

Challenge Model	**Sample**	***Vibrio aestuarianus* qPCR**	***Arcobacter* 23S qPCR**	***Malaciobacter marinus* qPCR**	***Vibrio aestuarianus***	***Malaciobacter marinus***
Injection	S1B-1	Negative	Positive (Ct=26.99)	Positive (Ct=36.49)	Negative	>300, confluent
	S1B-2	Negative	Positive (Ct=24.56)	Positive (Ct=34.58)	Negative	>300, confluent
	S2A-1	Positive (Ct=33.7)	Positive (Ct=26.29)	Positive (Ct=36.82)	Positive: 4.2 x 10^4 cfu's	>300, confluent
	S2A-3	Positive	Positive	Positive	Positive: 1.32	>300,

		(Ct=31.01)	(Ct=26.81)	(Ct=38.72)	x 10^6 cfu's	confluent
	S2B-1	Positive (Ct=28.82)	Positive (Ct=18.69)	Positive (Ct=38.71)	Positive: 1.28 x 10^5 cfu's	>300, confluent
	S2B-2	Negative	Positive (Ct=25.14)	Positive (Ct=37.69)	Positive: >3 x 10^6 cfu's	>300, confluent
Cohabitation-Fasted	S2C-1	Positive (Ct=31.3)	Positive (Ct=27.65)	Negative	Positive: >3 x 10^6 cfu's	n= 72
	S2C-4	Positive (Ct=34.41)	Positive (Ct=29.02)	Negative	Positive: >3 x 10^6 cfu's	n= 193
	S2C-5	Positive (Ct=31.5)	Positive (Ct=20.46)	Negative	Positive: >3 x 10^6 cfu's	n= 37
Cohabitation- Fed	S2D-1	Negative	Positive (Ct=29.92)	Negative	Positive: 3.2 x 10^6 cfu's	>300, confluent
	S2D-2	Positive (Ct=32.12)	Positive (Ct=23.83)	Negative	Positive: 3.06 x 10^6 cfu's	>300, confluent
	S2D-3	Positive (Ct=29.47)	Negative	Negative	Positive: 3.0 x 10^6 cfu's	>300, confluent
	S2D-4	Negative	Negative	Negative	Positive: >3 x 10^6 cfu's	>300, confluent
	S2D-6	Positive (Ct=27.27)	Negative	Negative	Positive: >3 x 10^6 cfu's	n= 110
	S2D-7	Positive (Ct=32.02)	Negative	Negative	Positive: >3x 10^6 cfu's	n= 30
	S2D-8	Negative	Positive (Ct=31.75)	Negative	Positive: >3 x 10^6 cfu's	>300, confluent
	S2D-9	Positive (Ct=32.77)	Positive (Ct=24.05)	Negative	Positive: >3 x 10^6 cfu's	>300, confluent


IRTA-19-132
Malaciobacter marinus CECT 7727T
Malaciobacter canalis F138-33T
IRTA-19-136
Malaciobacter molluscorum CECT 7696T
Malaciobacter neptunis F146-38T
Malaciobacter halophilus LA31BT
Malaciobacter mytili CECT 7386T
Halarcobacter anaerophilus DSM 24636T
Halarcobacter ebronensis F128-2T
Halarcobacter bivalviorum F4T
Halarcobacter mediterraneus F156-34
Halarcobacter ponticus F161-33T
IRTA-19-133
Halarcobacter salis F155-33T
Halarcobacter arenosus CAU 1517T
Malaciobacter pacificus SW028T
Arcomarinus aquaticus W112-28T
Poseidonibacter haliotis MA5T
Poseidonibacter lekithochrous LFT 1.7T
Poseidonibacter antarcticus SM1702T
Arcobacter peruensis PSE-93T
Poseidonibacter parvus LPB0137T
Arcobacter nitrofigilis CECT 7204T
Pseudarcobacter acticola KCTC 52212T
Pseudarcobacter venerupis F67-11T
Pseudarcobacter caeni RW17-10T
Pseudarcobacter suis CECT 7833T
Pseudarcobacter aquimarinus W63T
Pseudarcobacter cloacae SW28-13T
Pseudarcobacter defluvii SW28-11T
Pseudarcobacter ellisii F79-6T
Aliarcobacter butzleri LMG 10828T
Aliarcobacter lacus RW43-9T
Aliarcobacter hispanicus FW54
Aliarcobacter lanthieri AF1440T
Aliarcobacter vitoriensis F199T
Aliarcobacter porcinus CCUG 56899T
Aliarcobacter thereius LMG 24486T
Aliarcobacter skirrowii LMG 6621T
Aliarcobacter cryaerophilus LMG 9904T
Aliarcobacter trophiarum LMG 25534T
Aliarcobacter cibarius CECT 2703T
Aliarcobacter faecis AF1078T
Arcobacter vandammei R-73987
0.02

Supplementary Figure 2. Neighbor-Joining phylogenetic tree showing the relationship of the *Arcobacter*-like strains recovered from the mortality episode of adult Pacific oysters based on the concatenated sequence (1602bp) of *atpA* (563bp), *gyrB* (614bp), and *rpoB* (425bp). (Bootstrap percentage values based on 1,000 replications are shown at the nodes of the tree.) Bar indicates two substitutions per 100 nucleotides.

3.4 Study 4: Responses of *Mytilus galloprovincialis* to challenge with environmental isolates of the potential emerging pathogen *Malaciobacter marinus.*

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Fish and Shellfish Immunology is the official journal of the **International Society of Fish and Shellfish Immunology** (ISFSI). It presents studies on the basic mechanisms of both the specific and non-specific **defense systems**, the cells, tissues, and humoral factors involved, their dependence on environmental and intrinsic factors, response to **pathogens**, response to **vaccination**, and applied studies on the development of specific vaccines for use in the **aquaculture** industry. It is a **first quartile (Q1)** journal with an impact factor of **4.622** (2021).

Summary

The increasing occurrence of disease outbreaks results in mass mortality events in bivalves, particularly in Pacific oyster (*Crassostrea gigas*) and Mediterranean mussels (*Mytilus galloprovincialis*). The causes of these disease processes are multifactorial and involve both host and environmental factors. The outcome of the response to potential pathogens depends on complex interactions between the immune system of the host and the associated microbial communities. The paper investigated the responses of *Mytilus galloprovincialis* to *in vitro* challenges with two environmental isolates of the potential emerging pathogen *Malaciobacter marinus* isolated from *C. gigas* mortality episode in Ebro Delta, Spain, and previously characterized (study 3.3) in this thesis. The study found that both strains R1 (IRTA-19-131) and R2 (IRTA-19-132) of *M. marinus*, differing in the number of virulence genes, were moderately pathogenic to early larvae by studying the responses by *in vivo* challenge experiments. However, both strains of *M. marinus* decreased hemocyte lysosomal membrane stability (LMS) and stimulated extracellular defense responses (ROS production and lysozyme activity) in adult mussels. The interactions between mussel hemocytes and *M. marinus* were investigated in *in vitro* short-term experiments, and the results suggest their phagocytosis and subsequent lysosomal degradation. Overall, the results indicate the activation of both extracellular and intracellular immune defenses against *M. marinus* R1 (IRTA-19-131), resulting in a significant hemolymph bactericidal activity, with a large contribution of hemolymph serum. The study provides the first data on the potential pathogenicity of *Arcobacter* isolated from a shellfish mortality to bivalve larvae and adults, and on their interactions with the immune system of the host. While *M. marinus* seems to be sightly pathogenic for early larvae stages of mussel, based on the overall data, strain R1 utilized in the present work is among the less pathogenic to mussels

under laboratory conditions. However, the increased *in vivo* abundance of members of the *Arcobacter*-like spp. in stressed or diseased bivalve species, often in association with more pathogenic *Vibrio*, indicates that the interactions with the immune system of the host are extremely sensitive to perturbations by environmental factors or concomitant infections with pathogens, resulting in a strongly opportunistic behavior of *Arcobacter*. The study highlights the need for further research on the potential pathogenicity of these bacteria and their interactions with the immune system of the bivalve host.

UNIVERSITAT ROVIRA I VIRGILI
POTENTIAL IMPACT OF EMERGING BACTERIAL PATHOGENS ON SHELLFISH PRODUCTION
Faiz Ur Rahman

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Full length article

Responses of *Mytilus galloprovincialis* to challenge with environmental isolates of the potential emerging pathogen *Malaciobacter marinus*

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ABSTRACT

Bacteria of the *Arcobacter*-like spp. represent emerging foodborne zoonotic pathogens in humans and animals. Their increasing presence in seafood, suggesting higher occurrence in seawater due to marine pollution, is raising some environmental concern. Although *Arcobacter* is frequently detected in diseased oysters and stressed bivalve species, no data are available so far on its potential pathogenicity or interactions with the immune system of the bivalve host.

In this work, responses to challenge with two strains of *Malaciobacter marinus* IRTA-19-131 and IRTA-19-132, R1 and R2), isolated from adult *Crassostrea gigas* during a mortality event in 2019 in Spain, were investigated in the mussel *Mytilus galloprovincialis*.

In vivo experiments were performed in larvae (48 h post-fertilization), and in adult mussels at 24 h post-injection, in order to evaluate the pathogenicity for early developmental stages, and the hemolymph immune responses, respectively. Both R1 and R2 were moderately pathogenic to early larvae, with significant decreases in the development of normal D-veligers from 10^4 and 10^3 CFU/mL, respectively. In adults, both strains decreased hemocyte lysosomal membrane stability (LMS), and stimulated extracellular defense responses (ROS production and lysozyme activity).

The interactions between mussel hemocytes and *M. marinus* were investigated in *in vitro* short-term experiments (30–90 min) using the R1 strain (10^6–10^8 CFU/mL). R1 decreased LMS and induced lysosomal enlargement, but not cell detachment or death, and stimulated extracellular ROS production and lysozyme release, confirming *in vivo* data. Moreover, lysosomal internalization and degradation of bacteria were observed, together with changes in levels of activated mTor and LC3, indicating phagocytic activity. Overall, the results indicate the activation of both extracellular and intracellular immune defenses against *M. marinus* R1. Accordingly, these responses resulted in a significant hemolymph bactericidal activity, with a large contribution of hemolymph serum.

The results represent the first data on the potential pathogenicity of *Arcobacter* isolated from a shellfish mortality to bivalve larvae and adults, and on their interactions with the immune system of the host.

1. Introduction

In the last decades, aquacultured bivalves have been increasingly subjected to recurrent disease outbreaks resulting in mass mortality events. Although juveniles of the Pacific oyster (*Crassostrea gigas*) are the most severely affected, the occurrence of heterogeneous mortalities has been described in adults and other species such as mussels [1–3]. The causes of the disease processes that lead to mass mortality are not always evident; however, it is commonly accepted that these episodes are polymicrobial and multifactorial, involving both host and environmental factors (e.g. bivalves species, genetics and age, temperature, food availability, microbiota, pollutant exposure) [3]. Whatever the source and degree of infection, the outcome of the response to potential pathogens depends on complex interactions between the immune system of

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POTENTIAL IMPACT OF EMERGING BACTERIAL PATHOGENS ON SHELLFISH PRODUCTION
Faiz Ur Rahman

the host and the associated microbial communities [4]. Recent studies have reported the recurrent presence of bacteria of the genus *Arcobacter* in oysters (*C. gigas*) during mortality episodes [5,6]. Similar observations were made in laboratory experiments in oysters immune-compromised by OsHV-1 [7] or oysters and mussels exposed to temperature stress [8,9]. In mussels (*Mytilus galloprovincialis*) *exposed to nanoplastics, an increased abundance* of *Arcobacter* in hemolymph microbiome was observed [10].

Arcobacter spp. are recently regarded as emerging foodborne pathogens affecting both humans and animals, with the most common A. butzleri (reviewed in Ref. [11]). *Arcobacter* have been shown to inhabit very diverse environments, including sewage water [12]. Even though some *Arcobacter* species are believed to be free-living, waterborne organisms, and autochthonous to aquatic environments, they are also naturally present in bivalve microbiome [13,14]. While some species were found only in mussels (e.g. *A. mytili*) [15], the presence of others seems to be due to fecally contaminated freshwater inputs, as the presence of *A. butzleri* was correlated with the levels of *Escherichia coli* [16]. Since bivalves represent a cause of foodborne disease in humans, available literature mainly focuses on the detection of *Arcobacter* in edible species [11,14,16–18]. However, no data are available yet on the capacity of bivalves to respond to infection with *Arcobacter* and on the interactions with their immune system. Therefore, it is not clear if *Arcobacter* members can represent primary or opportunistic pathogens for different bivalves.

In this work, responses to challenge with two strains of *Malaciobacter marinus* isolated from *C. gigas* during a mortality event in 2019 in the Ebro delta in Spain (IRTA-19-131 and IRTA-19-132) were investigated in the Mediterranean mussel *M. galloprovincialis*. *In vivo* experiments were performed in larvae (48 h post-fertilization, hpf), and in adult mussels at 24 h post-injection (p.i.), in order to evaluate the pathogenicity for early developmental stages, and the hemolymph immune responses, respectively. The interactions with mussel hemolymph components and the mechanisms involved in the immune response were investigated in *in vitro* short-term experiments with IRTA-19-131. This work is part of a wider study on the epidemiology of *M. marinus* in the Ebro delta, along with the screening of other bacterial and viral pathogens, and on the *in vivo* pathogenicity of *M. marinus* in oysters.

2. Methods

2.1. Bacterial isolation and identification

Bacterial strains (IRTA-19-131 and IRTA-19-132) for challenge experiments were isolated from adult diseased oysters (*C. gigas*) collected during a mortality event that occurred in Fangar bay (40° 46.506′N; 0° 44.246′E, Ebro Delta, Spain) in June 2019. Briefly, aliquots (1 mL) of whole oyster tissue homogenates (from a pool of 5 oysters) were added with 9 mL of *Arcobacter* broth (Sigma-Aldrich, CM0965, Steinheim, Germany) supplemented with CAT [(C.A.T selective supplement: Cefoperazone-Amphotericin B-Teicoplanin) OxoidTM, SR0174E, Hampshire, UK] and 50% artificial seawater [19] and incubated aerobically at 23 °C in static conditions. After 48 h the tubes were vortexed for 10 s and aliquots of 100 μL were transferred to Marine Agar (MA) (Scharlab, 109352, Sentmenat, Spain) plates overlaid with 0.45 μm nitrocellulose membrane filters of (Millipore, Ref # HAWP04700, Tullagreen, Ireland) to retain larger bacteria. After 30 min, the filters were removed, and the plates were incubated aerobically at 23 °C for 48 h. *Arcobacter*-like spp. (i.e., small, transparent/translucent, beige, or off-white colonies) were sub-cultured on MA medium and incubated in the conditions mentioned above. Genomic DNA (gDNA) was extracted from bacterial isolates using QIAGEN DNeasy® Blood & Tissue Kit (QIAGEN, Ref # 69506, Hilden, Germany). Identification of the isolates was conducted by *Arcobacter* 23S genus specific qPCR and amplification and sequencing of the *rpoB* phylogenetic marker gene as previously described (GenBank accession numbers *rpoB*: OP441048 and OP441049) [15,20,21]. Two bacterial isolates were identified as belonging to the species *M. marinus* (IRTA-19-131 and IRTA-19-132).

2.2. Bacterial cultures and inoculum preparation

IRTA-19-131 and IRTA-19-132 strains (henceforth indicated as R1 and R2, respectively) were cultured in *Arcobacter* broth (conventional broth media containing peptone 18 g/L, yeast extract 1 g/L, NaCl 5 g/L modified with the addition of 50% artificial seawater-ASW) at 30 °C in a water bath with continuous agitation. After 24 h growth, the inoculum was centrifuged (4500×*g*, 20 min), and the pellet was resuspended to obtain a concentration of about 10^8 colony forming units CFU/mL (determined spectrophotometrically as an $Abs_{600} = 0.20$). MA was used throughout the experiments.

2.3. Effects of M. marinus R1 and R2 strains on mussel early larval development

Sexually mature mussels (*M. galloprovincialis*), 4–5 cm long, purchased in February 2021 from an aquaculture farm (La Spezia, Liguria, Italy) were acclimated in tanks with aerated artificial seawater (ASW), salinity 35 ppt (1 L/mussel) at 18 °C and utilized within 2 days for gamete collection [22]. The 48 h embryotoxicity assay was carried out in 96-microwell plates as in Balbi et al. (2019) [23]. At 30 min post-fertilization, aliquots of R1 and R2, from a 10^8 CFU/mL stock suspension, were tenfold serially diluted in ASW and added to fertilized eggs in each microwell to reach the desired nominal final concentrations (from 10^2 to 10^7 CFU/mL) in a 200 μL volume. Microplates were then incubated for 48 h at 18 °C ± 1 °C, with a 16:8 h light: dark photoperiod. All the procedures were carried out following ASTM [24]. At the end of incubation, each sample was fixed with buffered formalin (4%) and all larvae examined by optical microscopy using an inverted Olympus IX53 microscope (Olympus, Milano, Italy) at 40 × , equipped with a CCD UC30 camera and a digital image acquisition software (cellSens Entry). A D-shaped (straight hinge) larva with no protruding mantle was considered normal; if the typical stage at 48 hpf was not reached (trochophore or earlier stages) or when some developmental impairments were observed (concave, malformed or damaged shell, protruding mantle), the larva was considered malformed. The acceptability of test results was based on controls for a percentage of normal D-shell larvae >75% [24].

2.4. In vivo challenge of adult mussels with M. marinus R1 and R2 strains

Adult mussels (*M. galloprovincialis*), 4–5 cm long, were purchased in summer 2021 and acclimated in tanks with aerated artificial seawater (ASW) for 24 h, salinity 35 ppt (1 L/mussel) at 18 °C. Animals (20 mussels per condition) were injected into the posterior adductor muscle as previously described [23] utilizing 50 μL of live bacterial suspensions (*M. marinus* R1 or R2) containing 10^8 CFU/mL in PBS + NaCl (phosphate-buffered solution isotonic with hemolymph, PBS + NaCl: 2 mM KH_2HPO_4, 10 mM Na_2HPO_4, 3 mM KCl, 600 mM NaCl in distilled water, pH 7.4), in order to obtain a nominal concentration of 5×10^6 CFU/mL hemolymph. Control mussels were injected with PBS + NaCl. No mortality was observed during the experiments. At 24 h post-injection (p.i.), hemolymph was collected from the posterior adductor muscle of 4 pools of 4 mussels each. Hemocyte LMS, reactive oxygen species (ROS) production, and soluble lysozyme activity were determined in whole hemolymph samples from control and exposed groups. Hemolymph collection and assays were performed as previously described [23,25,26].

2.5. In vitro challenge of Mytilus hemocytes with M. marinus R1 strain

2.5.1. Hemolymph sampling and monolayer preparation

Using a sterile 1 mL syringe with an 18 G1/2″ needle, the

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hemolymph was extracted from the posterior adductor muscle. The hemolymph, with the needle prior removed, was filtered through sterile gauze and pooled at 18 °C. For hemocyte monolayers preparation, 20 μL drops of the whole hemolymph were deposited on microscope slides and the cells were let to adhere for 20 min before removing the excess of hemolymph. To obtain hemolymph serum-HS, the whole hemolymph was centrifuged at 100×*g* for 10 min, and the supernatant was sterilized through a 0.22 μm-pore filter. Hemocyte monolayers or whole hemolymph (according to the endpoint, see details in the next sections) were incubated with *M. marinus* R1 suspension suitably diluted in ASW or HS at different concentrations (10^6, 10^7, 10^8 CFU/mL) from 30 to 90 min, depending on the endpoint measured. Untreated samples in ASW or HS were run in parallel. Filter-sterilized ASW was utilized in all experiments.

2.5.2. Determination of functional parameters

All assays were carried out as previously described [23,25,26]. Lysosomal membrane stability (LMS) was evaluated in hemocytes monolayers by the Neutral Red Retention Time (NRRT). Hemocyte monolayers were pre-incubated for 30 min with different concentrations (10^6, 10^7, 10^8 CFU/mL) of *M. marinus* in ASW or HS. Samples were washed out and incubated with a neutral red (NR) (Sigma-Aldrich, Milan, Italy) solution (40 μg/mL in ASW). After 15 min, after removal of excess dye, a drop of ASW was added, and slides were observed under an optical microscope from time zero every 15 min, and the percentage of cells showing loss of dye from lysosomes in each field (10 fields each containing 8–10 cells) was evaluated until 50% of the cells showed sign of lysosomal leaking, i.e., the cytosol becoming red, and the cells rounded. All incubations were carried out at 18 °C.

For the determination of Lysozyme release and ROS production, aliquots of 400 μl of whole hemolymph were incubated with suspensions of *M. marinus* in ASW or HS (final concentrations 10^6 and 10^7 CFU/mL) for different times. Lysozyme activity in the extracellular medium after incubation with bacteria for 0, 30 and 60 min was evaluated spectrophotometrically at 450 nm using a suspension of *Micrococcus lysodeikticus* (15 mg/100 mL in 66 mM phosphate buffer, pH 6.4). Hen egg white (HEW) lysozyme was used as a standard and lysozyme activity was expressed as HEW lysozyme equivalents (U/mg protein/mL). Extracellular ROS generation was measured by the reduction of cytochrome *c*. Hemolymph samples were incubated for 30 min with a cytochrome *c* solution (75 μM ferricytochrome *c* in TBS (0.05 M Tris-HCl buffer, pH 7.6, containing 2% NaCl)). Cytochrome *c* in TBS was used as a blank. Samples were read at 550 nm against cytochrome *c* in TBS as a blank and the results were expressed as changes in OD per mg protein. All data are reported as percentage of control values. Protein content was determined using the Bradford method with bovine serum albumin (BSA) as a standard.

For *in vivo* experiments, functional parameters were evaluated directly on hemolymph samples after challenge with R1 or R2.

2.5.3. Giemsa staining

Hemocyte monolayers on microscope slides were incubated for 30 and 90 min with *M. marinus* R1. Samples were washed out, fixed in >50% methanol, and stained with a Giemsa kit (Diff-QuikTM, Medion Grifols Diagnostics AG, Switzerland), following the manufacturer instructions.

2.5.4. Electrophoresis and Western blotting

Hemocyte monolayers were incubated with bacterial suspensions in ASW (10^7 CFU/mL) at 18 °C for 30 and 90 min. Control samples were run in parallel. All procedures were carried out as previously described [26]. Supernatants from each culture dish were discarded and the hemocytes were lysed with 0.1 vol of ice-cold lysis buffer (50 mM Tris-HCl pH 7.8, 0.25 M sucrose, 1% SDS, 2 mM sodium orthovanadate, 0.1% Nonidet-P40, 5 mM M DTT) with protease inhibitors cocktail and phosphatase inhibitors cocktail (Roche, Basel, Switzerland). Samples were boiled for 4 min and then centrifuged for 20 min at 14,000×*g* to remove insoluble debris. Levels of target proteins were determined by SDS-PAGE and Western blotting using specific antibodies as previously described [26]. Samples were probed with rabbit polyclonal anti-LC3 (Sigma-Aldrich, L8918) antibodies (1:500), rabbit polyclonal mTor (phospho S2481) (www.abcam.com, ab137133) (1:1000) and rabbit polyclonal anti-actin antibody (Sigma-Aldrich, A5060) (1:500) as primary antibodies, and with horseradish peroxidase-conjugated goat anti-rabbit IgG (Invitrogen G21234) (1:3000) as secondary antibodies. Immune complexes were visualized using an enhanced chemiluminescence Western blotting analysis system (PURE-CL-Chemiluminescence Substrate Vilber) following the manufacturer's specifications. Western blotting films were digitized (Chemidoc-Biorad) and band optical densities (arbitrary units) were quantified using a computerized imaging system (QuantityOne).

2.5.5. Bactericidal activity

In vitro bactericidal activity of hemocyte monolayers was evaluated as previously described [23,25]. Hemocytes were incubated with *M. marinus* R1 (5×10^6 CFU/mL) at 18 °C in ASW or HS for different periods of time (0, 30 and 90 min). The bactericidal activity of HS was also evaluated. *M. marinus* in ASW was used as a control for bacterial growth over the experimental time. Immediately after the inoculum (T = 0) and after 30 and 90 min of incubation, supernatants were collected and hemocytes were lysed by adding filter-sterilized ASW supplemented with 0.05% Triton x-100 and by 10 s agitation. Supernatants and lysates were pooled and tenfold serial diluted in ASW. Aliquots (100 μL) of the diluted samples were plated onto MA. Samples of *M. marinus* suspensions in ASW or HS at the same concentrations were directly diluted and plated. After 48 h incubation at 30 °C, the number of CFU per hemocyte monolayer (representing live, culturable bacteria) was determined. Percentages of killing were determined in comparison to values obtained at zero time. The number of CFU in control hemocytes never exceeded 0.1% of those enumerated in experimental samples.

2.6. Data analysis

Data from *in vitro* and *in vivo* experiments on hemocyte and hemolymph functional parameters are the mean ± SD of at least 4 independent experiments, with each assay performed in triplicate. Statistical analyses were performed by the non-parametric Kruskal-Wallis test followed by Dunn's test ($p \leq 0.05$). Embryotoxicity test data, representing the mean ± SD of 4 independent experiments, carried out in 6 replicate samples in 96-microwell plates, were analyzed by ANOVA plus Tukey's post-test. Data from WB were analyzed by non parametric Mann-Whitney *U* test. All statistical calculations were performed using the GraphPad Prism version 7.03 for Windows, GraphPad Software, San Diego, CA, USA.

3. Results

3.1. Effects of M. marinus strains R1 and R2 on mussel early larval development

Fertilized eggs were exposed to either R1 or R2 in a wide concentration range (from 10^2 to 10^7 CFU/mL) and the percentage of normal D-larvae at 48 hpf was evaluated as previously described [5,23]. The results are reported in Fig. 1. Exposure to both strains moderately affected normal development, with significant effects for R1 from 10^4 CFU/mL and for R2 from 10^3 CFU/mL. At the maximal concentration tested, the effect of R1 was slightly stronger than that of R2 (with a −38% and −25% decrease in normal larvae, respectively). Interestingly, as shown by representative images, R1 mainly induced a delayed larval phenotype (immature veligers with protruding mantle and incomplete shell formation), whereas exposure to R2 mainly resulted in shell malformations (convex shell hinge).

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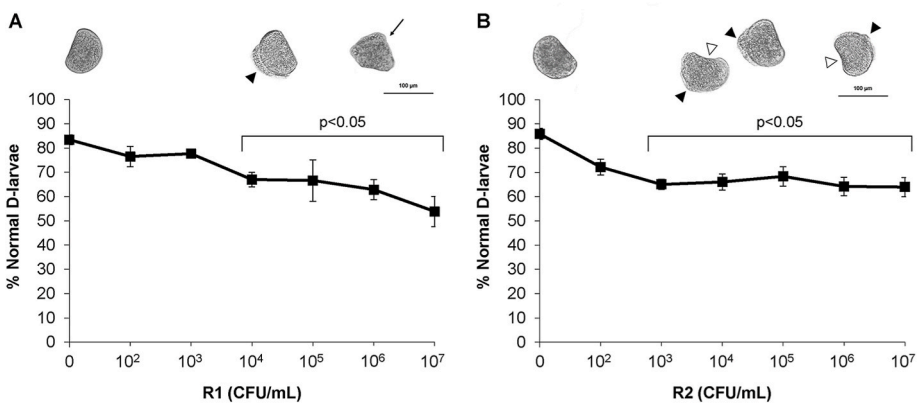


Fig. 1. Effects of different concentrations of *M. marinus* strains R1 and R2 on *M. galloprovincialis* larval development at 48 hpf. Percentage of normal D-shaped larvae with respect to controls after exposure to R1 (A) and R2 (B). Data, representing the mean ± SD of 4 experiments carried out in 96-multiwell plates, were analyzed by ANOVA plus Tukey's post-test ($p < 0.05$). Representative images of control larvae and of the main larval phenotypes (lateral view) observed in different experimental conditions at 48 hpf are reported (scale bar: 100 μm). Black ar = protruding mantle; arrow = immature larva (pre-veligers); white ar = convex hinge.

3.2. Effects of in vivo challenge of adult mussels with two M. marinus strains R1 and R2

The effects of *in vivo* challenge of mussels with *M. marinus* R1 and R2 were compared. Bacteria were injected into the posterior adductor muscle to reach a final nominal concentration of 5×10^6 CFU/mL in hemolymph. After 24 h p.i. the hemolymph was collected and functional immune parameters were evaluated (Fig. 2). Similar responses were observed with both strains. A significant decrease in LMS, evaluated as a marker of cellular stress, was observed (about −60 and −73% with respect to controls for R1 and R2, respectively) (Fig. 2A). This effect was accompanied by a large stimulation of extracellular ROS production (about +400% with respect to controls) (Fig. 2B) and of soluble lysozyme activity (+280% with respect to controls) (Fig. 2C) for both R1 and R2.

3.3. Effects of in vitro challenge of Mytilus hemocytes with M. marinus R1

The effects of *in vitro* short-term incubation (30 min) with different concentrations of the *M. marinus* strain R1 on hemocyte LMS were compared utilizing bacterial suspensions in different media, ASW or HS, and the results are reported in Fig. 3. A dose-dependent decrease in LMS was recorded in both media (Fig. 3A). Significant effects were observed from the lowest concentration tested (10^6 CFU/mL), with a −45% decrease in LMS with respect to control, until almost complete destabilization (>95%) at the highest concentration tested (10^8 CFU/mL). The effects of R1 on hemocyte LMS were compared with those of the R2 strain in similar experimental conditions. Since similar dose-dependent effects were observed (Fig. S1), subsequent *in vitro* experiments were carried out only with R1.

Early lysosomal changes induced by R1 in live hemocytes could be appreciated from the beginning of the NRR assay (see representative images in Fig. 3B obtained at time 0, after incubation with bacteria, washing and NR loading). In control samples (C), healthy hemocytes were characterized by the presence of numerous small NR loaded vesicles, representing individual lysosomes. In samples challenged with R1, lysosomal enlargement and fusion were observed at increasing concentrations of bacteria. Interestingly, at the end of the assay, when lysosomal destabilization was observed in >50% of hemocytes (corresponding to data shown in Fig. 3A), the cells remained fully adherent to the substrate, with no appreciable cell detachment and rounding typical of dying cells (Fig. S2).

B) Representative images of control hemocytes (C) and of hemocytes exposed to different concentrations of bacteria (10^6, 10^7, 10^8 CFU/mL in ASW) after loading with NR (time 0). Lysosomes are stained in red, nuclei and intact cytoplasm appear transparent (Scale bar: 10 μm).

Data on functional immune parameters, ROS production and lysozyme release, are reported in Fig. 4. Hemocyte exposure to *M. marinus* R1 suspensions in ASW did not elicit significant extracellular ROS production; however, in the presence of HS, a large increase in ROS was observed at both 10^6 and 10^7 CFU/mL (+350% to +480% respectively, with respect to controls) (Fig. 4A). Lysozyme activity showed small increases (+20-+50% with respect to controls), depending on the time of incubation (30 and 60 min) and exposure medium (Fig. 4B and C).

Hemocyte monolayers incubated with different concentrations of R1 (10^6–10^8 CFU/mL) in ASW for 30 or 90 min, were stained with Giemsa

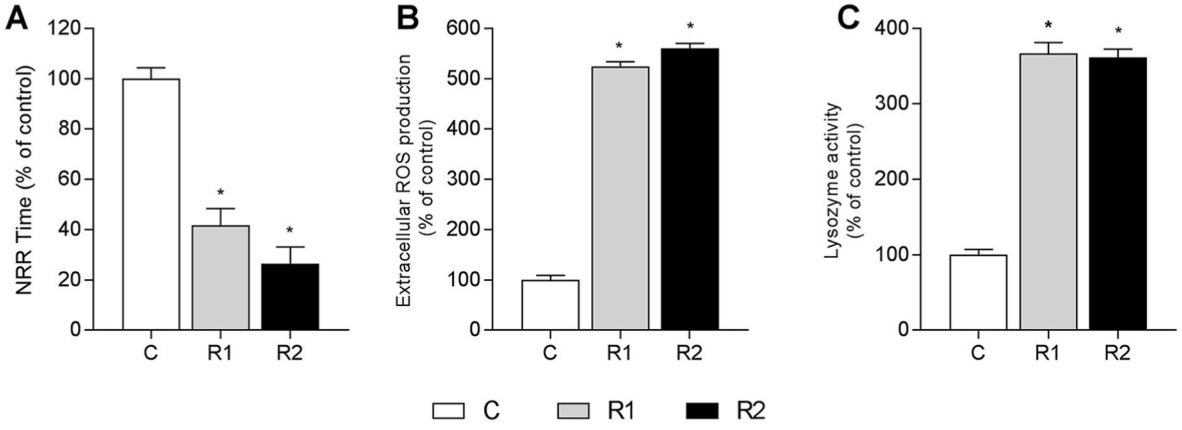


Fig. 2. *In vivo* effects of two *M. marinus* strains R1 and R2 on hemolymph parameters of *M. galloprovincialis*. Hemocyte lysosomal membrane stability-LMS (A), ROS production (B) and serum lysozyme activity (C) were evaluated in hemolymph sampled from mussels challenged with either R1 or R2 at 24 h p.i. Data are the mean ± SD of 4 independent experiments performed in triplicate. Statistical analyses were performed by non-parametric Kruskal–Wallis followed by Dunn's multiple comparisons test ($p < 0.05$).

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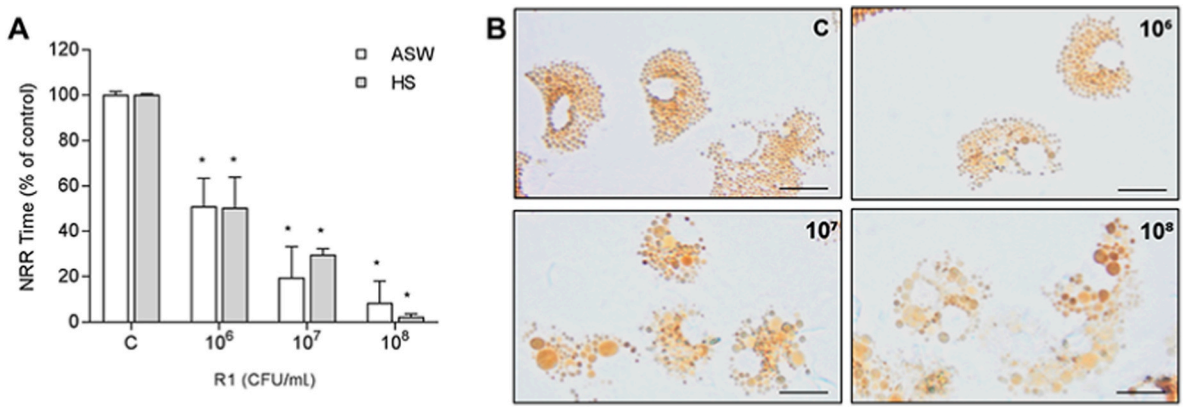


Fig. 3. ***In vitro*** **effects of** ***M. marinus*** **R1 strain on hemocyte lysosomal membrane stability-LMS.** A) LMS evaluated in different media (ASW or HS). Data, expressed as percent values with respect to controls and representing the mean ± SD of 4 experiments in triplicate. Statistical analyses were performed by non-parametric Kruskal–Wallis followed by Dunn's multiple comparisons test (* = $p < 0.05$).

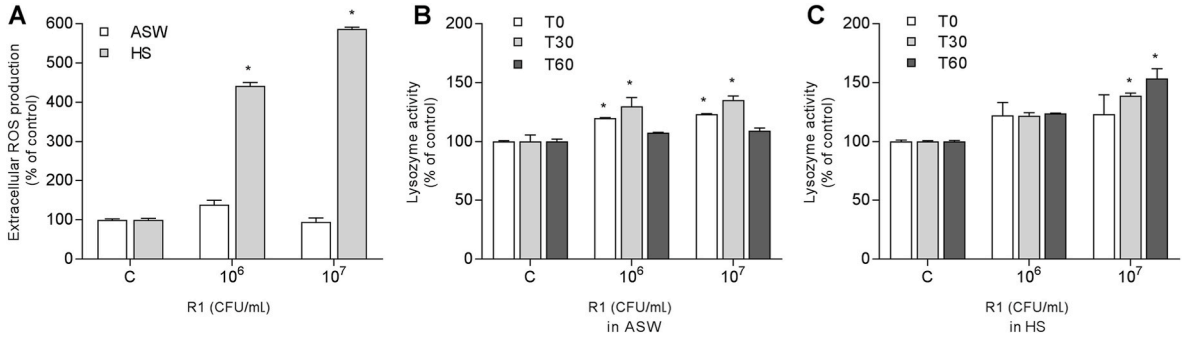


Fig. 4. ***In vitro*** **effects of** ***M. marinus*** **R1 on immune responses of** ***M. galloprovincialis*** **hemocytes**. Extracellular ROS production (A) and lysosomal enzyme release (B-C) were evaluated after incubation with R1 at 10^6 and 10^7 CFU/mL in ASW or HS. Hemocytes were incubated for different periods of time (30 min for ROS production and 30 and 60 min for lysozyme release). Data are the mean ± SD of at least 4 experiments performed in triplicate. Statistical analyses were performed by non-parametric Kruskal–Wallis followed by Dunn's multiple comparisons test (* = $p < 0.05$).

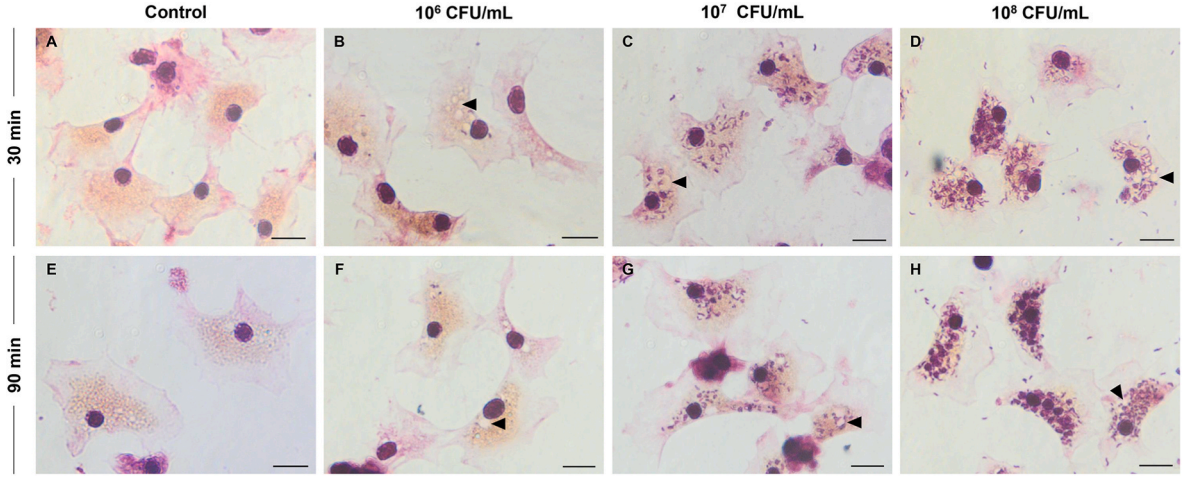


Fig. 5. Giemsa staining of control hemocytes and hemocytes incubated with different concentrations of *M. marinus* R1.
Hemocytes were incubated with R1 10^6, 10^7 and 10^8 CFU/mL in ASW for 30 (A-D) and 90 min (E-H). A and E) Controls, B and F) 10^6 CFU/mL, C and G) 10^7 CFU/mL and D and H) 10^8 CFU/mL. Black ars: enlarged lysosomes. Scale bar: 10 μm.

(Fig. 5). At both 30 and 90 min incubation, control hemocytes were well spread on the support, showing different cytoplasmic extensions, with the nuclei colored in dark violet, the cytosol in pink, and intracellular vacuoles and lysosomes (mainly located in the perinuclear region) in pink-yellowish (Fig. 5A and E). After 30 min incubation with R1 (Fig. 5B–D), an increasing number of bacteria were observed within the hemocytes, on cellular membranes and in the extracellular medium at increasing concentrations. Intracellular bacteria were mainly localized within enlarged lysosomal vesicles, indicating phagocytosis (black ar). Similar observations were made after 90 min incubation (Fig. 5, F-H), showing still adherent hemocytes with enlarged lysosomes. Moreover, at the highest concentration tested, enlarged vacuoles stained in dark

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violet were observed at both 30 and 90 min, containing bacteria. Similar results were obtained using bacterial suspensions in HS (not shown).

The possible mechanisms involved in *M. marinus* R1-induced lysosomal enlargement and internalization were investigated by evaluating the expression of the autophagic markers LC3 (microtubule-associated protein 1A/1B-light chain 3) and phosphorylated of mTor (mammalian target of rapamycin mTOR) by electrophoresis and Western Blotting [26], and the results are shown in Fig. 6. With regards to LC3, although the antibody can recognize both the LC3-I and LC-II forms, only the band corresponding to the lipidated LC3-II form could be identified in all samples (Fig. 6A). Although we previously identified both bands in mussel hemocytes collected in autumn (October) [26], in the present work, experiments were performed in mussels sampled in early summer. The presence of the LC3-II form only is often observed in different marine invertebrate models ([27] and refs therein); therefore, the differences in results obtained in hemocytes from mussels sampled at different times of the year may reflect seasonal variations in LC expression. The results of the densitometric analysis showed that challenge with R1 induced a large decrease in expression of LC3-II at both 30 and 90 min (about −60% with respect to controls at 30 min). A similar decrease was observed in the level of p-mTor (Fig. 6B). As previously reported [26], no changes in expression of LC3-II or p-mTor were observed in control samples at different times of incubation (not shown).

Finally, the bactericidal activity of mussel hemocytes towards *M. marinus* R1 (5×10^6 CFU/mL) was evaluated in the presence of ASW or HS, as well as in HS and ASW alone, at 30 and 90 min incubation, and data are expressed as the percentage of killed bacteria with respect time zero (Fig. 7). Hemocytes in ASW showed a significant and time-dependent bactericidal activity (20% and 75%, respectively, at 30 and 90 min). A similar effect was observed using HS alone (30% and 80%). Accordingly, the bactericidal activity was highest in samples of hemocytes in the presence of HS, in particular at shorter times of incubation (60% and 85%, at 30 and 90 min, respectively). No bacterial killing was observed in ASW alone.

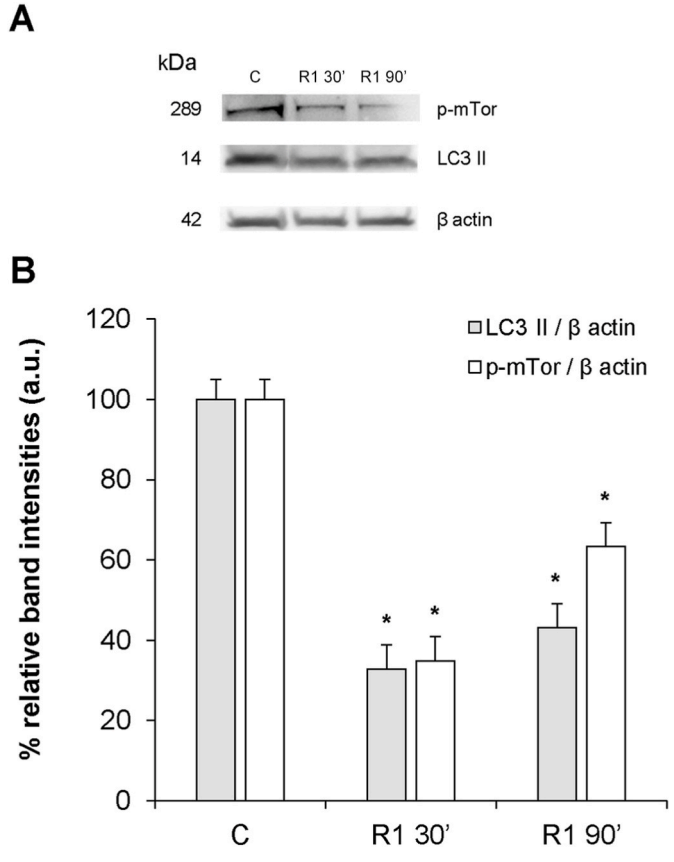


Fig. 6. Effects of *M. marinus* R1 on expression levels of LC3 and phosphorylated mTor in *M. galloprovincialis* hemocytes.
Expression of LC3-II and p-mTor in control hemocytes and hemocytes incubated for 30 and 90 min with R1 (10^7 CFU/mL). A) Representative blots, with β actin as a loading control. B) Densitometric analysis of blots from four independent experiments (mean ± SD). * $p \leq 0.05$, Mann-Whitney *U* test.

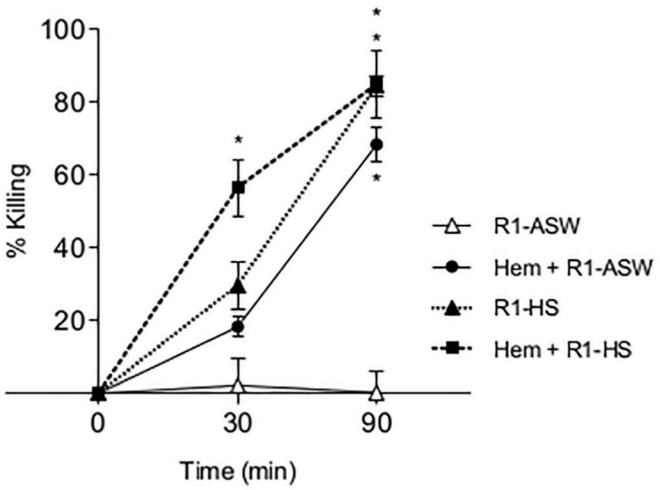


Fig. 7. *In vitro* bactericidal activity of mussel hemocytes and serum towards *M. marinus* R1.
Hemocytes were incubated for 30 and 90 min with *M. marinus* R1, at 5×10^6 CFU/mL resuspended in either ASW (Hem + R1-ASW) or hemolymph serum (Hem + R1-HS). In parallel, *M. marinus* R1 were incubated in ASW (R1-ASW) or HS (R1-HS) alone. At each time point, the number of viable, cultivable bacteria (CFU) per monolayer was evaluated, and the results are expressed as percentage values with respect to time zero. Statistical analyses were performed by non-parametric Kruskal–Wallis followed by Dunn's multiple comparisons test, * = $p < 0.05$, all treatments vs control (R1 in ASW).

4. Discussion

The results represent the first data on the interactions of environmental strains of *Arcobacter*-like spp. with marine bivalves. Two strains of *M. marinus* (IRTA-19-131 and IRTA-19-132) isolated from adult diseased oysters collected during a mortality event in the Ebro delta (Spain) were utilized.

Bivalve early developmental stages are likely to be more susceptible to pathogens than adults, since the immune system has not fully developed [28,29]. Therefore, we first tested the effects of *M. marinus* strains R1 and R2 in mussel early larvae utilizing the 48 h embryotoxicity assay. The results show that both strains affected normal larval development in a wide concentration range. Slight differences were observed between the two strains in terms of minimal effective concentrations (with significant effects observed from 10^3 to 10^4 CFU/mL, respectively, for R1 and R2), extent of the effects at the highest concentration tested (with R1 higher than R2), and main phenotypical changes (developmental delay for R1 and larval malformations for R2). Overall, the results indicate that both *M. marinus* strains are moderately pathogenic for early mussel larvae. In the same experimental conditions, stronger effects were observed with different pathogenic vibrios, such as *Vibrio coralliilyticus* [23], *V. tasmaniensis* LGP32, *V. bathopelagicus* sp. nov. [30].

Responses to challenge with *M. marinus* were then investigated in adult mussels *in vivo*, injected with either R1 or R2 (5×10^6 CFU/mL hemolymph) where functional hemolymph parameters were evaluated at 24 h p.i. The results show that both strains induced similar lysosomal stress in hemocytes (evaluated as a decrease in LMS); however, a large stimulation of extracellular defenses (ROS production and lysozyme activity) was observed, indicating that hemolymph components of adult

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mussels are able to mount an efficient defense response against *M. marinus* infection.

The mechanisms involved in immune interactions with *M. marinus* were subsequently investigated in *in vitro* short-term experiments in isolated hemocytes exposed for 30–90 min in the absence and presence of hemolymph serum (ASW or HS). Since both strains induced a similar concentration dependent effect on hemocyte LMS in a wide concentration range, we chose to utilize only R1 (10^6–10^8 CFU/mL) for further investigations. Lysosomal membrane destabilization was similar in the absence and presence of HS, and the effect was accompanied by lysosomal enlargement. However, no hemocyte rounding or detachment, indicating cell death, was observed. R1 induced a large and concentration dependent stimulation of extracellular ROS production in the presence of HS, but not in ASW, indicating a role for soluble components in the recognition of this strain and subsequent activation of membrane NADPH oxidase, the enzyme responsible for ROS production. R1 moderately stimulated the release of lysozyme release in both media, with differences in the extent and time course of the response. The results obtained *in vitro* thus confirm *in vivo* observations, showing that induction of lysosomal stress in the hemocytes was associated with stimulation of extracellular defense mechanisms.

Interactions of *M. marinus* with hemocytes were also investigated in Giemsa stained samples. After 30 min incubation, an increasing number of bacteria were visible inside the hemocytes at increasing concentrations, that were mainly localized within enlarged intracellular vesicles. Although optical microscopy does not allow for the identification of localization of bacteria, the results suggest their phagocytosis and subsequent lysosomal degradation.

With regards to the intracellular mechanisms involved in the response of hemocytes to R1 (lysosomal stress and enlargement, phagocytosis and lysosomal degradation of bacteria in the absence of cytotoxicity), the possible role of autophagic processes was investigated. In mussel hemocytes, we have previously demonstrated a protective role of autophagy against the pathogen *V. tapetis* [26]. *V. tapetis* induced a rapid stimulation of autophagy (induction of autophagosomes, and increase in autophagic markers LC3-II, p-mTor and p62), in the absence of phagocytosis and lysosomal degradation [26].

The results here obtained show that challenge with *M. marinus* resulted in contrasting effects on hemocyte autophagy markers. Although a decrease in mTor phosphorylation, indicating stimulation of autophagy, was observed, a similar effect was also recorded for LC3-II, the activated, lipidated form of LC3, whose increase is considered as an early marker of autophagy. Moreover, no changes in expression of p62 were observed (not shown). Overall, the results do not support an induction of the autophagic flux in mussel hemocytes by this strain of *M. marinus*. However, these data could be explained when considering by the multiple roles of mTor and LC3 in immune response. mTOR is a master kinase that also regulates lysosome structure and function and innate immunity; in macrophages, *V. vulnificus* induces mTor activation and inflammatory responses [31]. LC3 is also involved in LAP (LC3-associated phagocytosis), a form of non-canonical autophagy, best characterized in the clearance of pathogens by macrophages [32]. Briefly, in LAP, LC3 is conjugated to phagosome membranes while the bacterium is captured in the phagosome, thus acting as an innate immune response mechanism [32,33]. Unlike autophagy, LAP involves single-membrane structures, and a distinct rate of activation of LC3. Accordingly, in macrophages stimulated with *Candida albicans*, activated LC3 was increased at the peak of phagocytosis, and decreased at later times, when the process was completed [34]. In the present work, a similar condition is observed in mussel hemocytes during phagocytosis of *M. marinus* R1, indicating a decrease in LC3-II at 30–90 min, when the phagocytic process is well advanced or completed. These data provide the first indication for LAP, a unique process that links the ancient pathways of phagocytosis and autophagy in invertebrate immunocytes [35].

Finally, the bactericidal activity of hemolymph components was quantified by evaluating the percentage of killed bacteria in different experimental conditions, utilizing a concentration of R1 (5×10^6 CFU/mL) that induced both lysosomal stress and induction of extracellular defenses. After 30 min, a small bactericidal activity was shown by both hemocytes and HS alone (20% and 30% respectively). This activity could be ascribed to the extracellular release of ROS and enzymatic defenses (lysozyme) from hemocytes (Fig. 4) and to the direct effects of soluble serum components. A cumulative effect was observed for hemocytes in the presence of HS. Hemocytes alone showed a stronger bactericidal activity at longer incubation times (90 min), (up to 75%) this increase probably reflects the time needed for recognition of R1 in absence of HS, and consequent phagocytosis and lysosomal degradation. Maximal killing activity was observed with hemocytes in the presence of HS (85%). Although the components of HS involved in mediating immune defenses towards *M. marinus* strains were not identified, the results support the hypothesis that *Mytilus* hemolymph contains specific soluble factors that contribute to the general killing of many bacteria that are strongly pathogenic for other bivalve species [36,37].

The results obtained in *in vitro* experiments on the interactions between *M. marinus* strains and mussel hemocytes can be compared with previous data with other potentially pathogenic marine bacteria from the genus *Vibrio* tested in the same experimental conditions (Table 1). Among the most pathogenic vibrios for bivalves, *V. coralliilyticus* ATCC BAA-450 and *V. tasmaniensis* LGP32 induced strong cellular stress in the absence of efficient immune response and bactericidal activity [23,25]. Responses to *V. tapetis* consisted in absence or inhibition of immune responses [26]. *V. aestuarianus* 01/032 induced moderate stress in hemocytes where it was rapidly internalized and degraded by phagocytosis [25]. Environmental isolates of *V. parahaemolyticus* and *V. alginolyticus* elicited efficient immune responses, whereas *V. vulnificus* induced slower and minor responses with apoptosis [38]. The results obtained so far demonstrate that mussel hemocytes show different susceptibilities to different potential pathogens, according to the species and strain, reflecting specificities in pathogen recognition, binding and consequent activation of extracellular and intracellular defenses. Although the mechanisms involved in these specific interactions can be easily identified *in vitro*, the observed outcome of the response is generally observed also *in vivo* ([25], this work).

On the basis of the overall data, the *M. marinus* strain R1 utilized in the present work is among the less pathogenic to mussels under laboratory conditions. However, the increased *in vivo* abundance of members of the *Arcobacter*-like spp. in stressed or diseased bivalve species, often in association with more pathogenic *Vibrio* [4], indicates that the interactions with the immune system of the host are extremely sensitive to perturbations by environmental factors or concomitant infections with pathogens, resulting in a strongly opportunistic behavior of *Arcobacter*.

Table 1
Summary of *in vitro* data on immune parameters in *M. galloprovincialis* hemocytes challenged with *M. marinus* R1 and different *Vibrio* (10^7 CFU/mL) in the presence of HS. The parameters reported include: lysosomal membrane stability (LMS), extracellular ROS production and lysozyme release, expressed as % of control values. Bactericidal activity is reported as % of killed bacteria with respect to the inoculum.

	LMS	ROS	Lyso. act	Killing
M. marinus R1	↓↓↓	↑↑↑	↑	↑↑↑
V. coralliilyticus[a]	↓↓↓	≈	nd	≈
V. tasmaniensis[b]	↓↓↓	nd	↑↑	≈
V. aestuarianus[b]	≈	nd	↑	↑
V. tapetis[c]	↓	↓↓	≈	≈
V. parahaemolyticus[d]	↓↓	↑	↑	nd

[a] *V. coralliilyticus* ATCC BAA-450 [23].
[b] *V. tasmaniensis* LGP32 and *V. aestuarianus* 01/032 [25].
[c] *V. tapetis* LP2 [26].
[d] *V. parahaemolyticus* Conero 80 [38]. nd: not determined. ↓: decreased; ↑: increase; ≈ no effect.

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In this light, the role of the non-virulent microbiota should not be neglected, as non-virulent *Vibrio* strains have the potential to dramatically increase the host damages caused by virulent strains [4]. Data from co-challenge experiments with pathogenic and opportunistic bacteria may help understanding these interactions. Moreover, edible bivalves can vehiculate *Arcobacter*-like species up to humans, as shown by a recently reported case of human bacteremia induced by *M. mytili* [39]. Further information is also needed on the interactions of *Arcobacter* strains with the immune system of other bivalves; in particular research is in progress on the interactions between *M. marinus* strains and immune system of the oyster *C. gigas*.

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CRediT authorship contribution statement

Manon Auguste: Conceptualization, Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Faiz Ur Rahman:** Methodology, Formal analysis. **Teresa Balbi:** Conceptualization, Data curation, Software. **Martina Leonessi:** Data curation, Formal analysis, Investigation. **Caterina Oliveri:** Data curation, Formal analysis, Investigation. **Grazia Bellese:** Formal analysis. **Luigi Vezzulli:** Conceptualization, Resources, Supervision, Writing – review & editing. **Dolors Furones:** Resources, Supervision, Writing – review & editing. **Laura Canesi:** Conceptualization, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

None.

Data availability

No data was used for the research described in the article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2022.09.048.

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4. GENERAL DISCUSSION

GENERAL DISCUSSION

General Discussion

The gram-negative rod-shaped *Arcobacter*-like species are ubiquitously present in nature, and they have been isolated from a wide range of ambiances, from humans, terrestrial animals, food, vegetables, dairy products, fresh water, marine water, and shellfish. Many terrestrial species like *A. butzleri, A. cryaerophilus, A. skirrowii,* etc. have been associated with human and animal diseases, moreover, *A. butzleri* has been classified as zoonotic pathogen by the International Commission on Microbiological Specifications for Foods (ICMSF, 2002). In recent years, it has been reported that high prevalence is encountered in shellfish (Salas-Massó et al., 2019), which can cause illness in the consumers as shellfish are consumed raw or poorly cooked (Collado and Figueras, 2011; Levican et al., 2014; Ferreira et al., 2016, 2017; Salas-Massó et al., 2016). Therefore, exploring the role/s of *Arcobacter spp* in the marine environment is an open field.

4.1 Development of new enrichment broth for isolation of *Arcobacter*-like spp.

There is none standard media for the isolation of *Arcobacter*-like spp. from different sources and the importance of such media has been underlined by Collado and Figueras (2011) in their review. For isolating *Arcobacter*-like spp. from marine environment, Salas-Massó et al. (2016) developed the Arcobacter-CAT-broth + 2.5% NaCl and found that this enrichment broth was a good medium for recovering the marine related *Arcobacter*-like spp. since they could report many new species e.g., *Malaciobacter canalis,* using it. However, we still need more knowledge on the diversity of *Arcobacter*-like spp. in the marine environment to help studying its relationship with marine invertebrates, like shellfish (Salas-Massó et al., 2016; Mizutani et al., 2019). Therefore, as stated in the first objective of this thesis, an improved enrichment medium was developed for the isolation and culturing of *Arcobacter*-like spp. from the marine environment (**3.1 Study 1**). Of the 24 enrichment broth media formulation tested, we included

the conventional Arcobacter broth used in previous studies (Collado et al., 2009; Salas-Massó et al., 2016) for comparison purposes with artificial sea salt, and indeed *Arcobacter* isolates grew more in broth supplemented with 50% artificial seawater (ASW) (Salinity = 16.9 ppt) than in the previously developed Arcobacter broth + 2.5% NaCl (salinity = 27.8 ppt). For the isolation of a microorganism from its natural environment, microbiologists use the strategy of mimicking its main characteristics, both environmental parameters and nutrient's profile. The failure in isolating and growing bacteria in the laboratory can be attributed to not providing *in vitro* conditions mirroring the original environment e.g., nutrients, pH, temperature, osmotic pressure etc. (Kabore et al., 2019). Since the occurrence of *Arcobacter*-like spp. in both shellfish and marine waters have been attributed to its indigenous marine environment (Miller et al., 2007; Fernandez-Piquer et al., 2012; King et al., 2012), **study 1 (Section 3.1)** attempted to emulate such habitat both by providing the salts and osmolarity conditions of seawater and the nutrient components of the oyster tissue. Seawater contains NaCl as a major salt component, but it also contains other macro and micronutrients e.g., Magnesium, Potassium, Sulphates, Calcium, Boron, Fluoride etc. that play a major role in their growth as nutrients, electron transporter, osmotic agents, and/or as co-factors for the enzymes proper functioning (Tyler et al., 1960; Joint et al., 2010; Pinet, 2019). Following this rationale, the Arcobacter broth + 50% ASW was developed, proving to have a significantly higher strain recovery rate and growth yield than its predecessor Arcobacter broth + 2.5% NaCl (Salas-Massó et al., 2016) for marine *Arcobacter*-like spp. and, therefore, it was used in the following studies of study **2** to **4 (Section 3)**.

4.2 Epidemiological survey of pathogens in *Crassostrea gigas*

In the next objective (**Study 2**) we sampled pacific oyster (*C. gigas*) from shellfish farms in Ebro Delta, where mortalities were being observed recurrently, to study further the etiology of the episodes, focusing on infectious agents. These mortalities can be very severe, causing more

than 80% mortality in *C. gigas* juveniles (Carrasco et al., 2017), affecting oyster production in the area. To obtain contrasted samples (mortality vs non-mortality), samplings were carried out during the mortality episodes occurring from November 2018 to June 2020, while contrasted non-mortality samples were also studied when available. As expected, the known viral and bacterial pathogens prevalent in the area: OsHV-1 virus, *V. aestuarianus* and *V. splendidus* clade respectively, were detected during mortality episodes.

An important pathogen for *C. gigas* that we targeted was *V. aestuarianus* (Garnier et al., 2007; 2008; Vezzulli et al., 2015; Degremont et al., 2020), since information of it in the area was scarce, but it had been reported in the Ebro Delta in 2016, associated with adult *C. gigas* mortality (73% prevalence) in 2016 and 30% in 2017 in spat (Carrasco et al., 2018; Lasa et al., 2019). Both detections were by means of molecular methods, such as 16S rRNA profiling by next generation sequencing (Lasa et al., 2019). To our knowledge, *V. aestuarianus* had not been isolated before in *C. gigas* from the Ebro Delta until the present study (**Study 2**). The *V. aestuarianus* strains from the Ebro Delta outbreaks, were included in a comparative study with other isolates across Europe, where they were found to be clones of *V. aestuarianus* subsp. *francensis* (Mesnil et al., 2022). *V. aestuarianus* was detected in a modest number of samples (31.58%) over the study period, but only during mortality episodes of *C. gigas*. The observed prevalence ranged from 3.33% to 30%, which was comparatively lower than the previously reported in the area by Lasa et al. (2019). Neither isolation nor detection of *V. aestuarianus* was obtained from the non-mortality episode samples, however it was detected and isolated from local commercial oysters used in the challenge experiment 2 of **Study 3,** forming part of the bacterial background, and it was suspicious of causing mortalities during challenge experiment 2, which is coherent with other studies in *C. gigas* (Azema et al., 2016; 2017; Alvarez-Munoz et al., 2019; Lasa et al., 2019; Degremont et al., 2020; 2021).

Regarding *V. splendidus*, species of this clade were present in both mortality and non-mortality episode samples. Moreover, *V. splendidus* clade was detected in 78.95% of the sampling episodes, with a prevalence ranging from 3.33% to 96.67% independently of the mortality rates. Previous studies suggest that these bacteria could be found naturally associated with *C. gigas* as a non-pathogenic commensal which could turn into a pathogenic form when the host immune system is weaken or when the oysters are infected by another pathogen (Nasfia et al., 2015; Bruto et al., 2018; Oyanedel et al., 2023).

The next group of bacteria studied from the polymicrobial infections of *C. gigas* from Ebro Delta was *Arcobacter*-like spp. This group includes more than 33 species and have been found in the hemolymph and tissue microbiota of *C. gigas* (Lokmer et al., 2015; 2016; King et al., 2019) and in other shellfish (Levican et al., 2014; Salas-Massó et al., 2016; Mudadu et al., 2021). Different reports from the Ebro Delta, indicated that shellfish could be a reservoir for *Arcobacter*-like spp. (Collado et al., 2009; Levican, 2013; Fernandez et al., 2015). In this study, they have been found in 94.74% sampling episodes, with a prevalence from 6.7% up to 90% in different samples. As occurs with *V. splendidus* clade, *Arcobacter*-like spp. comprises several species, thus, considering the high prevalence found, we aimed to further characterize the isolates up to specie level, so, study their relationship with *C. gigas*.

4.3 Genetic diversity of *Arcobacter*-like isolates in the *C. gigas*

The diversity of *Arcobacter* genotypes found from the *C. gigas* with the ERIC-PCR method (Number of different ERIC-PCR patterns / Number of isolates) was 26% representing a total of 37 strains identified among 141 investigated isolates. An almost similar i.e., 24.8%, genetic diversity has been previously reported by Levican (2013) in his thesis, from the shellfish samples collected in the same study area (Ebro Delta). While in other matrixes like chicken meat the reported diversity ranged from 11.1% to 59.9%, whereas in beef meat was 30% (Aydin

et al., 2007), 44.8% in broiler and chicken carcasses (Van Driessche and Houf, 2008) and 65% in bovine carcasses (De Smet et al., 2010). The *rpoB* gene sequencing and the phylogenetic analysis revealed that 80.14% (113/141) of the isolates from mortality episodes belonged to *Malaciobacter marinus*, showing a similarity of 99.3% to 99.7% by BLASTN with the sequence of this species deposited at (NCBI database). This indicated that this species could have a role in the mortality events of *C. gigas*, and, therefore, it was further studied at functional level, *in vivo* and in *vitro* (**Study 3 and 4**). *M. marinus* was the only dominant *Arcobacter*-like spp. isolated from *C. gigas* in mortality samples (80.14%), while the remaining 19.86% isolates of *Arcobacter*-like spp. belonged to several potentially novel *Arcobacter*-like species. However, since the prevalence of those isolates were not linked to shellfish mortalities, they were not further characterized in the present study, nonetheless, it shows the potential of discovering new strains of *Arcobacter*-like spp. in the marine environment using the cultivation media supplemented with sea salt.

4.4 *In vivo* bacterial challenge experiments in *C. gigas*

Functional studies followed, such as *in vivo* challenge experiments in *C. gigas* to study the potential pathogenicity of *Malaciobacter marinus* both in single and in dual challenge experiments (**Study 3**). The oysters, farmed in the Ebro Delta, used in the experiments were representative of the commercial stocks. Conversely, they were not specific pathogen free (no SPF), thus they could be the carriers of different pathogens including those under this study (Le Roux et al., 2016). The *in vivo* challenge experiments were performed both by injecting the *M. marinus* strains into *C. gigas* (10% and 25% mortality) and by cohabitating with the infected oyster (50% and 90% mortalities), mimicking closer naturals conditions. These same methods were used by several researchers to study the pathogenicity of different bacterial pathogens such as *V. splendidus*, *V. aestuarianus*, *V. harveyi* and OsHV-1, in *C. gigas* and other shellfish by studying the pathogenicity progression by injecting the pathogen or

cohabitating with the diseased / infected shellfish, where the mortalities were initially recorded but the animals got resistant after few days (Gay et al., 2004; Gagnaire et al., 2007; Saulnier et al., 2010; Decker & Saulnier, 2011; Petton et al., 2013; 2015; Azema et al., 2015; Travers et al., 2017; Andree et al., 2021). Although the experimental *C. gigas* stocks were negative for known pathogens i.e., OsHV-1, *V. aestuarianus* prior to the challenge, the postmortem examination of the animals from the cohabitation experiments, showed that *V. aestuarianus* was present in the moribund animals along with experimental *M. marinus*. Therefore, the observed mortalities in the challenge experiments could be attributed to the known pathogen, *V. aestuarianus*, and potentially also to *M. marinus* since both microbes were detected / isolated from the moribund challenged animals. This was also found later in experiment 4 that the dual challenge with both these bacteria resulted in comparatively higher mortalities than one. It has been reported by several authors that polymicrobial infection has more severe effects than those of a single pathogen in *C. gigas* infected by *V. crassostreae* and *Shewanella* sp. (Lemire et al., 2015) and OsHV-1 and *V. aestuarianus* at the same time (Azema et al., 2016). In our work, in the dual challenge **(Study 3)**, *C. gigas* mortality was higher (90%) when injected with both *M. marinus* and *V. aestuarianus*, although *V. aestuarianus* alone also induced very high mortality (75%), with no significant difference with the dual challenge ($p<0.05$), in contrast with the low or no mortality attributable to *M. marinus* in our experimental set up. In a previous study by Lemire et al. (2015) it was observed that *C. gigas* challenged only with *V. crassostreae* caused only 5% mortality but when dually challenged with *Shewanella* sp. the mortality significantly increased to 60%. Since this was the first time that both *V. aestuarianus* and *M. marinus* were tested together *in vivo*, there is no comparative references; however, the results show that mortality could worsen in a polymicrobial scenario when oysters are coinfected. To our knowledge, this is the first study of pathogenicity of *M. marinus* in *C. gigas* (shellfish) and, it is the first report of *V. aestuarianus* isolated from Ebro Delta and tested *in vivo* in *C. gigas*,

showing a mortality pattern like that reported in the south of France (Mesnil et al., 2022), from where many oyster shipments come from in the Delta.

The *in vivo* challenges experiments generated interesting first preliminary results on *M. marinus* and in *V. aestuarianus*, in this case the first isolate in our area. However, the impossibility of working with Specific Pathogen Free (SPF) animals-imposed limitations in the scope of the *in vivo* approach, since our experimental animals were depurated commercial stocks and, therefore, they were harboring microbiomes and potentially containing the microorganism under study. Moreover, despite the preliminary screening of the experimental animals, to test the prevalence of *V. aestuarianus* and *M. marinus*, that was limited by the sensibility of the qPCR methods on tissue samples, which was low for *V. aestuarianus* and very poor for *M. marinus* with our developed Mm-rpoB qPCR assay. Saulnier et al. (2009) observed the detection limit for *V. aestuarianus* to be 1.6 x 10^2 cell/mg which corresponds to 1.6 cells of *V. aestuarianus*. Saulnier et al. (2009) also observed the inhibition in qPCR assay when oyster DNA was spiked artificially with *V. aestuarianus* DNA. The background loads of both bacteria were better assessed by cultivation, followed by performing qPCR of the colonies, by which the methods accomplished higher sensitivity. Therefore, the bacterial background results were obtained, inevitably, once the experiment had begun. However, the approach was an interesting one to preliminary assess pathogenicity, considering the need for screening bacterial load by cultivation.

4.5 *In vitro* challenge with shellfish hemolymph

Following, in our **Study 4**, we evaluated *in vitro* the interaction of *M. marinus* (IRTA-19-131 and IRTA-19-132) isolated from *C. gigas* during mortality episodes in Ebro Delta with shellfish hemolymph since this approach is more controllable even losing the whole animal system. Since *Arcobacter*-like spp. has regularly been detected in moribund oysters and other

bivalves (Lokmer et al., 2015; 2016; King et al., 2019; Lasa et al., 2019), but no data on their pathogenicity was available, neither on their interaction with the immune cells of the hosts it was planned to run the *in vitro* assays in both *M. galloprovincialis* and *C. gigas*, along the lines developed and optimized in previous work with the Mediterranean mussel, *Mytilus galloprovincialis* (Balbi et al., 2013; 2018; 2019; Pezzati et al., 2015; Auguste et al., 2019; 2020). It was found that the *M. marinus* sp. gave a very significant stress to the hemocytes in both shellfish species i.e., *M. galloprovincialis* and *C. gigas*, as the lysosomal membrane stability (LMS) of the hemocytes from both shellfish spp. was significantly reduced by these bacteria as observed in previous studies by Blabi et al. (2013; 2018; 2019) and Pezzati et al., (2015). Other extracellular parameters tested, such as reactive oxygen species (ROS) production and lysozyme production were also increased in response to these *M. marinus* strains in *M. galloprovincialis*. The bacterial killing of *M. marinus* by *M. galloprovincialis* hemocytes with serum increased with long incubation times (**Study 4**), initially, at 30 minutes of incubation, killing was 20%, and increased to 75% after 90 minutes which indicates that *M. galloprovincialis* serum helps its hemocytes in killing of *M. marinus*. While in *C. gigas* the opposite happened (39.5% killing initially, and 26% at 90 minutes) and the *M. marinus* overcome the killing by *C. gigas* hemocytes and start growth again in these hemocytes. In previous studies by Pezzati et al. (2015) they observed 27% killing of *V. aestuarianus* 01/032 by *M. galloprovincialis* hemocytes which later increased to 61% after 90 minutes of incubation. This may be because the mussel hemolymph contains several components that can help bacterial pathogen killing, while *C. gigas* lack those components (Pezzati et al., 2015; Canesi et al., 2016). Apart from LMS and bacterial killing assays it was also found that *M. marinus (*strains IRTA-19-131 and IRTA-19-132) are moderate pathogens to the early larvae of *M. galloprovincialis* as the larvae didn't develop after infection with these *M. marinus* strains. The

overall preliminary results of **Study 4** indicated that these *M. marinus* strains conferred stress on the immune system of these shellfish species i.e., *M. galloprovincialis* and *C. gigas*.

Finally, the polymicrobial infection that has been observed in **Study 2** of this thesis can be attributed to the different roles of the pathogens studied i.e., OsHV-1, *V. aestuarianus*, and the potential pathogenic *M. marinus* (*Arcobacter*-like spp.) that they play in *C. gigas* mortality. Although this is a preliminary study, it highlights the prevalence of *M. marinus* in mortality episodes of *C. gigas* and raises questions for further studies to demonstrate its role. Epidemiological evidence on the prevalence of *Arcobacter*-like spp. in *C. gigas*, and specifically of *M. marinus* in mortalities have been provided, complemented with diagnostic tools, which set the basis and can be of assistance for future work. The studies should be expanded to other important shellfish and bivalves, such as mussels and clams etc. to find the role of these bacteria in their health. Specific care should be taken in exploring further the role of *M. marinus* in both *C. gigas* and mussels, specifically during polymicrobial infections. Opening also to study the bacterial interactions *in vitro*, quorum sensing phenomena. Moreover, a specific and highly sensitive screening tool is advised for this species while studying the polymicrobial infections, experimental infections, and mortalities in shellfish.

GENERAL DISCUSSION

5. CONCLUSIONS

CONCLUSIONS

Conclusions

1. The results from the supplementation with artificial seawater (ASW) to the Arcobacter enrichment broth media, followed by culturing on marine agar media, indicated that shellfish-associated *Arcobacter*-like species growth was significantly higher in Arcobacter broth + 50% ASW and in the same media supplemented with lyophilized oysters. Consequently, the study successfully developed an improved enrichment broth for enhancing the isolation and growth of *Arcobacter*-like species from marine and shellfish matrices, highlighting the importance of mimicking the original environmental conditions. Therefore, the Arcobacter broth + 50% ASW provides an efficient tool for screening marine *Arcobacter*-like species from shellfish and marine environment and should help to enhance the description of diversity of species obtained from shellfish.

2. The enrichment of Arcobacter broth + 50% ASW and culture protocol by using a lower temperature than previous i.e., 23 ºC resulted in the first isolation of *Malaciobacter marinus* from the moribund oysters mainly, during several mortality episodes in Ebro Delta Spain, this finding would assist epidemiological studies.

3. The mortality episodes of farmed *C. gigas* that occurred in the Ebro Delta during the study period, were linked with polymicrobial infections that involved Ostreid Herpes virus 1 (OsHV-1), *Vibrio aestuarianus*, *V. splendidus* clade and *Arcobacter*-like spp. It was found that OsHV-1 and *V. aestuarianus* were found in *C. gigas* only in mortality episodes samples, while *V. splendidus* clade and *Arcobacter*-like spp. were present in both mortality and non-mortality episodes. *V. aestuarianus* was detected mainly in adult oysters in summer / warm periods.

4. As, *Arcobacter*-like bacteria consists of many different species, the further genetic profiling and phylogenetic analysis of the isolates revealed that mainly *Malaciobacter marinus* was present in the samples from mortality episodes, while it was not isolated from samples of non-mortality episodes. Moreover, *M. marinus* was detected in all mortality episodes, in association with both Ostreid Herpes virus 1 (OsHV-1), *Vibrio aestuarianus*.

5. Very important biological material was acquired from moribund oysters, including the first isolation of *V. aestuarianus* and several isolates of *Arcobacter*-like spp., which were essential for the further functional work performed in the thesis, including the *M. marinus* isolates used to perform the *in vivo* and *in vitro* functional studies, as well as the development of the specific PCR.

6. A *Malaciobacter marinus* specific conventional PCR and qPCR assays were developed using *M. marinus* specific Mm-rpoB primer set, as a tool to assist identification in epidemiological and functional studies.

7. Considering the strong association observed in the epidemiological work, between mortality of *C. gigas* and *M. marinus* prevalence, the role of *M. marinus*, as a potential pathogen for *C. gigas*, was studied by *in vivo* bacterial challenge experiments in *C. gigas* both in single and dual pathogen experiments, with *V. aestuarianus*. While the pathogenicity of *V. aestuarianus* seems evident, *M. marinus* needs further work, with Specific Pathogen Free (SPF) *C. gigas*, to discriminate properly the effect of *M. marinus* in a bacteriological background free of *V. aestuarianus*. Nevertheless, to our

knowledge, this is the first study where both *M. marinus* and *V. aestuarianus*, isolated during mortality episodes of *C. gigas* in the Ebro Delta, have been tested *in vivo*, to assess its role as potential bacterial pathogens for *C. gigas*.

8. The *in vitro* challenge experiments of *M. marinus* with *C. gigas* hemocytes, showed that it stressed the *C. gigas* hemocytes and although initially it was killed by *C. gigas* hemocytes but later it can proliferate in their presence, indicating that it has the potential to evade the Pacific oyster immune system.

9. The responses of *Mytilus galloprovincialis* to *in vitro* challenges with two environmental isolates of the potential emerging pathogen *M. marinus* isolated from *C. gigas* mortality episode in Ebro Delta, Spain, found that they were moderately pathogenic to early larvae.

10. *M. marinus* decreased hemocyte lysosomal membrane stability (LMS) and stimulated extracellular defense responses (ROS production and lysozyme activity) in adult mussels. Overall, the results indicate the activation of both extracellular and intracellular immune defenses against *M. marinus* R1 (IRTA-19-131), resulting in a significant hemolymph bactericidal activity, with a large contribution of hemolymph serum. These results point towards an opportunistic nature of *M. marinus* in *M. galloprovincialis*.

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ANNEX

ANNEX

ANNEX I

Summary of bacterial culturing and related data from oyster samples collected from November 2018 to June 2020 in Ebro Delta, Spain.

NA= Data not Available/not collected

F= Fangar Bay

A= Alfacs Bay

D; Diploid oyster

T= Triploid Oyster

ANNEX

Sample #	Sampling Period	Origin	Age/ Mean Size (mm)	Ploidy	n=	Mortality %	*V. aestuarianus* average CFU's	*Standard Deviation (±SD)*	*V. splendidus* clade average CFU's	*Standard Deviation (±SD)*	*Arcobacter*-like spp. average CFU's	*Standard Deviation (±SD)*	*M. marinus* average CFU's	*Standard Deviation (±SD)*
1	2018/Nov/29	A	Adult (94.43)	D	30	0%	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
2	2018/Nov/30	F	Adult (79.38)	D	30	19%	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
3	2019/Mar/28-A	IRTA	Juvenile (46.23)	D	30	0%	0.00E+00	0.00E+00	7.10E+02	1.20E+02	3.10E+03	2.00E+02	0.00E+00	0.00E+00
4	2019/Mar/28-B	A	Juvenile (46.53)	D	30	17%	0.00E+00	0.00E+00	3.60E+02	2.94E+01	0.00E+00	0.00E+00	3.60E+03	4.97E+02
5	2019/Mar/28-C	F	Juvenile (47.17)	D	30	51%	0.00E+00	0.00E+00	2.20E+03	2.69E+02	0.00E+00	0.00E+00	2.17E+04	4.75E+03
6	2019/May/24	F	Adult (53.34)	D	30	8.94%	0.00E+00	0.00E+00	3.20E+03	3.39E+02	0.00E+00	0.00E+00	1.20E+04	1.80E+03
7	2019/May/29	F	Adult (55.97)	D	30	0%	0.00E+00	0.00E+00	1.70E+03	4.90E+02	9.00E+02	1.51E+02	0.00E+00	0.00E+00
8	2019/Jun/27*	F	Spat (23.34)	D	29	33%	NA	NA	NA	NA	NA	NA	NA	NA
9	2019/Jun/28-A	F	Adult (84.2)	D	30	17.50%	1.48E+02	3.73E+01	0.00E+00	0.00E+00	0.00E+00	0.00E+00	2.31E+04	6.30E+03
10	2019/June/28-B	F	Adult (73.3)	T	30	50.80%	2.07E+04	7.34E+03	0.00E+00	0.00E+00	0.00E+00	0.00E+00	6.95E+03	2.83E+03
11	2020/Feb/25-A**	F	Adult (87.83)	T	20	13%	0.00E+00	0.00E+00	6.75E+01	8.29E+00	0.00E+00	0.00E+00	5.50E+04	9.41E+03
12	2020/Feb/25-B**	F	Adult (79.98)	T	20	29%	0.00E+00	0.00E+00	5.20E+02	8.22E+01	0.00E+00	0.00E+00	2.51E+04	4.63E+03
13	2020/Feb/26	F	Adult (86.66)	D	30	0%	0.00E+00	0.00E+00	2.40E+02	5.32E+01	1.03E+04	4.35E+03	0.00E+00	0.00E+00
14	2020/Apr/21	F	Adult (98.4)	T	30	31%	6.30E+03	1.99E+03	1.70E+02	2.00E+01	0.00E+00	0.00E+00	5.70E+03	1.70E+03
15	2020/Apr/22	F	Adult (74.04)	T	30	29%	9.60E+03	2.70E+03	2.10E+02	5.89E+01	0.00E+00	0.00E+00	1.61E+04	8.17E+03
16	2020/Jun/04-A**	F	Adult (100.6)	T	20	68%	3.40E+05	2.94E+04	2.78E+03	1.30E+02	0.00E+00	0.00E+00	1.01E+05	4.95E+04
17	2020/Jun/04-B	F	Adult (102.1)	T	30	37%	0.00E+00	0.00E+00	6.25E+04	1.05E+04	0.00E+00	0.00E+00	6.72E+04	2.83E+04
18	2020/Jun/17-A	F	Adult (95.68)	D	30	7%	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	9.07E+04	2.77E+04
19	2020/June/17-B	F	Adult (99.23)	D	30	31%	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	3.42E+04	6.97E+03

ANNEX II

Summary of *Vibrio aestuarianus* culturing and related data from oyster samples collected from November 2018 to June 2020 in Ebro Delta, Spain.

NA= Data not Available/not collected

F= Fangar Bay

A= Alfacs Bay

D; Diploid oyster

T= Triploid Oyster

ANNEX

Sample #	Sampling Period	Origin	Age/ Mean Size (mm)	Ploidy	n=	Mortality %	*V. aestuarianus* average CFU's	Standard Deviation (±SD)	Pool 1	Pool 2	Pool 3	Pool 4	Pool 5	Pool 6	Average	Standard Deviation
1	2018/Nov/ 29	A	Adult (94.43)	D	30	0%	0.00E+00	0.00E+00	0	0	0	0	0	0	0	0
2	2018/Nov/ 30	F	Adult (79.38)	D	30	19%	0.00E+00	0.00E+00	0	0	0	0	0	0	0	0
3	2019/Mar/ 28-A	IRTA	Juvenile (46.23)	D	30	0%	0.00E+00	0.00E+00	0	0	0	0	0	0	0	0
4	2019/Mar/ 28-B	A	Juvenile (46.53)	D	30	17%	0.00E+00	0.00E+00	0	0	0	0	0	0	0	0
5	2019/Mar/ 28-C	F	Juvenile (47.17)	D	30	51%	0.00E+00	0.00E+00	0	0	0	0	0	0	0	0
6	2019/May/ 24	F	Adult (53.34)	D	30	8.94%	0.00E+00	0.00E+00	0	0	0	0	0	0	0	0
7	2019/May/ 29	F	Adult (55.97)	D	30	0%	0.00E+00	0.00E+00	0	0	0	0	0	0	0	0
8	2019/Jun/2 7*	F	Spat (23.34)	D	29	33%	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
9	2019/Jun/2 8-A	F	Adult (84.2)	D	30	17.50%	1.48E+02	3.73E+01	0	0	162	185	97	0	148	37.264818
10	2019/June/ 28-B	F	Adult (73.3)	T	30	50.80%	2.07E+04	7.34E+03	24700	12900	0	14500	0	307 00	20700	7336.2116
11	2020/Feb/2 5-A**	F	Adult (87.83)	T	20	13%	0.00E+00	0.00E+00	0	0	0	0	0	0	0	0
12	2020/Feb/2 5-B**	F	Adult (79.98)	T	20	29%	0.00E+00	0.00E+00	0	0	0	0	0	0	0	0
13	2020/Feb/2 6	F	Adult (86.66)	D	30	0%	0.00E+00	0.00E+00	0	0	0	0	0	0	0	0
14	2020/Apr/2 1	F	Adult (98.4)	T	30	31%	6.30E+03	1.99E+03	5400	0	0	3600	730 0	890 0	6300	1991.2308
15	2020/Apr/2 2	F	Adult (74.04)	T	30	29%	9.60E+03	2.70E+03	0	0	6900	12300	0	0	9600	2700
16	2020/Jun/0 4-A**	F	Adult (100.6)	T	20	68%	3.40E+05	2.94E+04	310000	380000	0	330000	NA	NA	340000	29439.203
17	2020/Jun/0 4-B	F	Adult (102.1)	T	30	37%	0.00E+00	0.00E+00	0	0	0	0	0	0	0	0
18	2020/Jun/1 7-A	F	Adult (95.68)	D	30	7%	0.00E+00	0.00E+00	0	0	0	0	0	0	0	0
19	2020/June/ 17-B	F	Adult (99.23)	D	30	31%	0.00E+00	0.00E+00	0	0	0	0	0	0	0	0

ANNEX III

Summary of *Vibrio splendidus* clade culturing and related data from oyster samples collected from November 2018 to June 2020 in Ebro Delta, Spain.

NA= Data not Available/not collected

F= Fangar Bay

A= Alfacs Bay

D; Diploid oyster

T= Triploid Oyster

ANNEX

Sample #	Sampling Period	Origin	Age/ Mean Size (mm)	Ploidy	n=	Mortality %	*V. splendidus* clade average CFU's	Standard Deviation (±SD)	Pool 1	Pool 2	Pool 3	Pool 4	Pool 5	Pool 6	Average	±SD
1	2018/Nov/29	A	Adult (94.43)	D	30	0%	0.00E+00	0.00E+00	0	0	0	0	0	0	0	0
2	2018/Nov/30	F	Adult (79.38)	D	30	19%	0.00E+00	0.00E+00	0	0	0	0	0	0	0	0
3	2019/Mar/28-A	IRTA	Juvenile (46.23)	D	30	0%	7.10E+02	1.20E+02	830	0	590	0	0	0	710	120
4	2019/Mar/28-B	A	Juvenile (46.53)	D	30	17%	3.60E+02	2.94E+01	0	0	320	390	370	0	360	29.43920
5	2019/Mar/28-C	F	Juvenile (47.17)	D	30	51%	2.20E+03	2.69E+02	1830	2460	2310	0	0	0	2200	268.7005
6	2019/May/24	F	Adult (53.34)	D	30	8.94%	3.20E+03	3.39E+02	3000	0	2800	3300	0	3700	3200	339.1165
7	2019/May/29	F	Adult (55.97)	D	30	0%	1.70E+03	4.90E+02	2140	1220	0	1640	2370	1130	1700	490.1836
8	2019/Jun/27*	F	Spat (23.34)	D	29	33%	#DIV/0!	#DIV/0!	NA	NA	NA	NA	NA	NA	NA	NA
9	2019/Jun/28-A	F	Adult (84.2)	D	30	17.50%	0.00E+00	0.00E+00	0	0	0	0	0	0	0	0
10	2019/June/28-B	F	Adult (73.3)	T	30	50.80%	0.00E+00	0.00E+00	0	0	0	0	0	0	0	0
11	2020/Feb/25-A**	F	Adult (87.83)	T	20	13%	6.75E+01	8.29E+00	60	70	60	80	NA	NA	67.5	8.291562
12	2020/Feb/25-B**	F	Adult (79.98)	T	20	29%	5.20E+02	8.22E+01	630	540	400	510	NA	NA	520	82.15838
13	2020/Feb/26	F	Adult (86.66)	D	30	0%	2.40E+02	5.32E+01	180	160	260	310	280	250	240	53.22906
14	2020/Apr/21	F	Adult (98.4)	T	30	31%	1.70E+02	2.00E+01	190	0	0	0	150	0	170	20
15	2020/Apr/22	F	Adult (74.04)	T	30	29%	2.10E+02	5.89E+01	0	270	0	0	130	230	210	58.87840
16	2020/Jun/04-A**	F	Adult (100.6)	T	20	68%	2.78E+03	1.30E+02	2650	2910	0	0	NA	NA	2780	130
17	2020/Jun/04-B	F	Adult (102.1)	T	30	37%	6.25E+04	1.05E+04	0	73000	52000	0	0	0	62500	10500
18	2020/Jun/17-A	F	Adult (95.68)	D	30	7%	0.00E+00	0.00E+00	0	0	0	0	0	0	0	0
19	2020/June/17-B	F	Adult (99.23)	D	30	31%	0.00E+00	0.00E+00	0	0	0	0	0	0	0	0

ANNEX IV

Summary of *Arcobacter*-like spp. (Other than *Malaciobacter marinus*) culturing and related data from oyster samples collected from November 2018 to June 2020 in Ebro Delta, Spain.

NA= Data not Available/not collected

F= Fangar Bay

A= Alfacs Bay

D; Diploid oyster

T= Triploid Oyster

ANNEX

Sample #	Sampling Period	Origin	Age/ Mean Size (mm)	Ploidy	n=	Mortality %	*Arcobacter*-like spp. average CFU's	Standard Deviation (±SD)	Pool 1	Pool 2	Pool 3	Pool 4	Pool 5	Pool 6	Average	Standard Deviation
1	2018/Nov/29	A	Adult (94.43)	D	30	0%	0.00E+00	0.00E+00	0	0	0	0	0	0	0	0
2	2018/Nov/30	F	Adult (79.38)	D	30	19%	0.00E+00	0.00E+00	0	0	0	0	0	0	0	0
3	2019/Mar/28-A	IRTA	Juvenile (46.23)	D	30	0%	3.10E+03	2.00E+02	0	0	3300	0	2900	0	3100	200
4	2019/Mar/28-B	A	Juvenile (46.53)	D	30	17%	0.00E+00	0.00E+00	0	0	0	0	0	0	0	0
5	2019/Mar/28-C	F	Juvenile (47.17)	D	30	51%	0.00E+00	0.00E+00	0	0	0	0	0	0	0	0
6	2019/May/24	F	Adult (53.34)	D	30	8.94%	0.00E+00	0.00E+00	0	0	0	0	0	0	0	0
7	2019/May/29	F	Adult (55.97)	D	30	0%	9.00E+02	1.51E+02	1090	0	0	890	720	0	900	151.217
8	2019/Jun/27*	F	Spat (23.34)	D	29	33%	#DIV/0!	#DIV/0!	NA	NA	NA	NA	NA	NA	NA	NA
9	2019/Jun/28-A	F	Adult (84.2)	D	30	17.50%	0.00E+00	0.00E+00	0	0	0	0	0	0	0	0
10	2019/June/28-B	F	Adult (73.3)	T	30	50.80%	0.00E+00	0.00E+00	0	0	0	0	0	0	0	0
11	2020/Feb/25-A**	F	Adult (87.83)	T	20	13%	0.00E+00	0.00E+00	0	0	0	0	0	0	0	0
12	2020/Feb/25-B**	F	Adult (79.98)	T	20	29%	0.00E+00	0.00E+00	0	0	0	0	0	0	0	0
13	2020/Feb/26	F	Adult (86.66)	D	30	0%	1.03E+04	4.35E+03	11700	6900	7800	8300	19400	7700	10300	4346.262
14	2020/Apr/21	F	Adult (98.4)	T	30	31%	0.00E+00	0.00E+00	0	0	0	0	0	0	0	0
15	2020/Apr/22	F	Adult (74.04)	T	30	29%	0.00E+00	0.00E+00	0	0	0	0	0	0	0	0
16	2020/Jun/04-A**	F	Adult (100.6)	T	20	68%	0.00E+00	0.00E+00	0	0	0	0	0	0	0	0
17	2020/Jun/04-B	F	Adult (102.1)	T	30	37%	0.00E+00	0.00E+00	0	0	0	0	0	0	0	0
18	2020/Jun/17-A	F	Adult (95.68)	D	30	7%	0.00E+00	0.00E+00	0	0	0	0	0	0	0	0
19	2020/June/17-B	F	Adult (99.23)	D	30	31%	0.00E+00	0.00E+00	0	0	0	0	0	0	0	0

ANNEX V

Summary of *Malaciobacter marinus* culturing and related data from oyster samples collected from November 2018 to June 2020 in Ebro Delta, Spain.

NA= Data not Available/not collected

F= Fangar Bay

A= Alfacs Bay

D; Diploid oyster

T= Triploid Oyster

ANNEX

Sample #	Sampling Period	Origin	Age/ Mean Size (mm)	Ploidy	n=	Mortality %	*M. marinus* average CFU's	Standard Deviation (±SD)	Pool 1	Pool 2	Pool 3	Pool 4	Pool 5	Pool 6	Average	Standard Deviation
1	2018/Nov/29	A	Adult (94.43)	D	30	0%	0.00E+00	0.00E+00	0	0	0	0	0	0	0	0
2	2018/Nov/30	F	Adult (79.38)	D	30	19%	0.00E+00	0.00E+00	0	0	0	0	0	0	0	0
3	2019/Mar/28-A	IRTA	Juvenile (46.23)	D	30	0%	0.00E+00	0.00E+00	0	0	0	0	0	0	0	0
4	2019/Mar/28-B	A	Juvenile (46.53)	D	30	17%	3.60E+03	4.97E+02	0	4300	0	3300	3200	0	3600	496.65
5	2019/Mar/28-C	F	Juvenile (47.17)	D	30	51%	2.17E+04	4.75E+03	28900	23200	17800	15300	0	23300	21700	4750.15
6	2019/May/24	F	Adult (53.34)	D	30	8.94%	1.20E+04	1.80E+03	13100	0	0	10900	14300	9700	12000	1802.77
7	2019/May/29	F	Adult (55.97)	D	30	0%	0.00E+00	0.00E+00	0	0	0	0	0	0	0	0
8	2019/Jun/27*	F	Spat (23.34)	D	29	33%	#DIV/0!	#DIV/0!	NA	NA	NA	NA	NA	NA	NA	NA
9	2019/Jun/28-A	F	Adult (84.2)	D	30	17.50%	2.31E+04	6.30E+03	29700	27900	25700	19500	10900	24900	23100	6303.43
10	2019/June/28-B	F	Adult (73.3)	T	30	50.80%	6.95E+03	2.83E+03	0	4500	11000	4100	0	8200	6950	2832.40
11	2020/Feb/25-A**	F	Adult (87.83)	T	20	13%	5.50E+04	9.41E+03	43000	68000	59000	50000	NA	NA	55000	9407.44
12	2020/Feb/25-B**	F	Adult (79.98)	T	20	29%	2.51E+04	4.63E+03	24200	17900	28800	29500	NA	NA	25100	4628.71
13	2020/Feb/26	F	Adult (86.66)	D	30	0%	0.00E+00	0.00E+00	0	0	0	0	0	0	0	0
14	2020/Apr/21	F	Adult (98.4)	T	30	31%	5.70E+03	1.70E+03	7500	7900	3300	3900	6100	5500	5700	1697.05
15	2020/Apr/22	F	Adult (74.04)	T	30	29%	1.61E+04	8.17E+03	10100	14500	26400	9100	28200	8300	16100	8174.14
16	2020/Jun/04-A**	F	Adult (100.6)	T	20	68%	1.01E+05	4.95E+04	103000	181000	56000	64000	NA	NA	101000	49492.42
17	2020/Jun/04-B	F	Adult (102.1)	T	30	37%	6.72E+04	2.83E+04	84000	112000	62000	31000	35000	79000	67166.667	28292.61
18	2020/Jun/17-A	F	Adult (95.68)	D	30	7%	9.07E+04	2.77E+04	88000	42000	129000	75000	112000	98000	90666.667	27686.74
19	2020/June/17-B	F	Adult (99.23)	D	30	31%	3.42E+04	6.97E+03	23000	36000	31000	44000	37000	0	34200	6968.5006

ANNEX VI

Proportion of *Malaciobacter marinus* and other *Arcobacter*-like species isolated from pacific oyster *Crassostrea gigas* in the Ebro Delta, Spain.

Oyster Age	**Time Period**	**Mortality %**	**Other *Arcobacter*-like spp. %**	***M. marinus* sp. %**	**Number of isolates of Other *Arcobacter*-like spp.**	***Number of isolates of M. marinus* sp.**
Juvenile	2019/Mar/28-A	0	100	0	3+/3	0+/3
	2019/Mar/28-B	17	0	100	0+/4	4+/4
	2019/Mar/28-C	51	42.86	57.14	3+/7	4+/7
Adult	2018/Nov/29	0	0	0	0+/0	0+/0
	2018/Nov/30	19	0	0	0+/1	0+/1
	2019/May/24	8.94	0	100	0+/12	12+/12
	2019/May/29	0	100	0	5+/5	0+/5
	2019/Jun/28-A	17.5	29.73	70.27	11+/37	26+/37
	2019/Jun/28-B	50.8	11.76	88.24	2+/17	15+/17
	2020/Feb/25-A	13	0	100	0+/4	4+/4
	2020/Feb/25-B	29	0	100	0+/4	4+/4
	2020/Feb/26	0	100	0	3+/3	0+/3
	2020/Apr/21	31	0	100	0+/7	7+/7
	2020/Apr/22	29	0	100	0+/6	6+/6
	2020/Jun/04-A	68	0	100	0+/4	4+/4
	2020/Jun/04-B	37	0	100	0+/4	4+/4
	2020/Jun/17-A	7	0	100	0+/11	11+/11
	2020/Jun/17-B	31	33.33	66.67	4+/12	8+/12


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