Antibacterial activity of marine sea cucumber and oyster against *Staphylococcus aureus and Vibrio*

Inaugural-Dissertation

to obtain the academic degree

Doctor rerum naturalium (Dr. rer. nat.)

submitted to the Department of Biology, Chemistry, Pharmacy

of Freie Universität Berlin

by

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Berlin 2023

This thesis was conducted between March 2020 and December 2023, under the supervision of Prof. Dr. Jens Rolff (Freie Universität Berlin). The work was carried out at Freie Universität Berlin.

1st Reviewer: Prof. Dr. Jens Rolff

2nd Reviewer: Prof. Dr. Dino Peter Mcmahon

Date of defense: 23.09.2024

To My Mother

And

My Dear Father,

I'm honored to share with you the culmination of my academic journey - my dissertation. While you're no longer with us, your unwavering belief in me continues to inspire every achievement. This work stands as a tribute to your enduring influence and the values you instilled in me. Your guidance remains my compass, guiding me through this academic endeavor and life itself.

Thank you for being my guiding light.

With love and eternal gratitude

DECLARATION OF INDEPENDENCE

Herewith I certify that I have prepared and written my thesis independently and that I have not used any sources and aids other than those indicated by me.

Berlin, 5th December, 2023

Noushin Arfatahery

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Antibakterielle Aktivität von Seegurke und Auster gegen *Staphylococcus aureus* und *Vibrio*

ZUSAMMENFASSUNG

Die Ozeane und Meere beherbergen einen großen Teil des Lebens auf der Erde: 71 % der Erdoberfläche bestehen aus Meerwasser, während nur 2,5 % trinkbares Süßwasser sind. Ozeanisches Plankton, Algen und bestimmte Bakterien tragen wesentlich zu diesem großen Anteil bei. Die marinen Lebensräume werden in Küstenlebensräume und offene Ozeane unterteilt, wobei die Küstenlebensräume nur sieben Prozent der Meeresoberfläche einnehmen. Die untersuchten Organismen reichen vom mikroskopisch kleinen Phytoplankton und Zooplankton bis hin zu Walen im offenen Ozean. Wirbellose Tiere sind ein wichtiger Bestandteil des Lebens im Meer und liefern Nahrung, Medizin und Rohstoffe. Die Weiterentwicklung von aus Wirbellosen gewonnenen Verbindungen ist eine therapeutische Perspektive für die biomedizinische Forschung. Die Entdeckung von Naturstoffen aus dem Meer hat neun zugelassene Medikamente hervorgebracht, und 12 befinden sich derzeit in der klinischen Erprobung, die größtenteils aus Sammlungen wirbelloser Meerestiere aus tropischen Flachwasser-Ökosystemen stammen. Die weltweite Fischproduktion hat erhebliche Auswirkungen auf die Volkswirtschaften, da sie 17 % des von der Weltbevölkerung konsumierten tierischen Eiweißes liefert und der Gesundheit dient. Die Aquakultur leistet einen wichtigen Beitrag zur globalen Fischereiindustrie.

Die Seegurke ist ein wirbelloses Meerestier, das in Ostasien aufgrund seiner vielfältigen biologischen und pharmakologischen Eigenschaften verzehrt wird, darunter krebshemmende, antiangiogene, gerinnungshemmende, entzündungshemmende, blutdrucksenkende, antimikrobielle, antithrombotische, antioxidative und antitumorale Eigenschaften. Der Persische Golf ist ein biodiverses Ökosystem, in dem *Holothuria leucospilota* die am häufigsten geerntete Seegurke ist. Allerdings wurden die Holothuria-Arten des Persischen Golfs bisher nur in 20

Artikeln untersucht. Frühere Untersuchungen haben gezeigt, dass hohe Konzentrationen von Holothuria-Verbindungen eine schwache antibakterielle Wirkung haben. Seegurkenproteinhydrolysate sind eine gesunde und zuverlässige Alternative zu künstlichen Lebensmittelkonservierungsmitteln. *Staphylococcus aureus* ist einer der am weitesten verbreiteten Erreger von Lebensmittelvergiftungen, und das Auftreten von Antibiotikaresistenzen bei *S. aureus* ist ein Problem für die öffentliche Gesundheit.

In Kapitel II untersuchte ich anhand der Extraktion bioaktiver Verbindungen aus der Seegurke *Holothuria leucospilota* die antibakterielle Wirkung auf MRSA- und *Enterotoxin-produzierende Staphylococcus aureus*-Stämme (SEASA, SEBSA), die bestimmt wurde. Zur Bestimmung der antimikrobiellen Aktivität wurden drei Methoden verwendet: Diskusdiffusionsexperimente, die minimale bakterizide Konzentration (MBC) und die minimale Hemmkonzentration (MIC). Die Ergebnisse zeigen, dass Methanol- und Chloroformextrakte das Wachstum aller getesteten Isolate bei MIC-Konzentrationen von bis zu 100 mg/ml hemmen. In Konzentrationen von etwa 100 mg/ml zeigte der Chloroformextrakt eine bakterizide Wirkung gegen SEBSA. In Konzentrationen unter 100 mg/ml ist der Extrakt auch bakterizid gegen MRSA und SEBSA. Der Methanolextrakt zeigte die größte antibakterielle Aktivität. Daher ist Seegurkenextrakt ein vielversprechender Kandidat für die Entdeckung neuer antimikrobieller Wirkstoffe; es sind jedoch noch umfangreiche Forschungsarbeiten erforderlich, um die aktiven Bestandteile von *Holothuria leucospilota* aus dem Persischen Golf zu isolieren und zu identifizieren. Ich habe herausgefunden, dass Seegurkenextrakte antimikrobielle Eigenschaften besitzen können. Meine Ergebnisse unterstützen die Idee, dass Seegurkenextrakte eine gesunde und zuverlässige Alternative zu künstlichen Lebensmittelkonservierungsmitteln darstellen, was ihre antimikrobielle Aktivität verdeutlicht.

Vibriose ist eine potenziell tödliche Infektion, die durch *Vibrio*-Arten verursacht wird, die in Meeres- und Ästuar-Ökosystemen weit verbreitet sind und beim Menschen Hautinfektionen und Magen-Darm-Erkrankungen verursachen können. *Vibrio spp*. nimmt aufgrund des Klimawandels zu, und die Pazifische Auster *Crassostrea gigas* ist ein geeignetes Modell für die Untersuchung der Wechselwirkungen zwischen *Vibrio* und Wirt bei der Krankheitsdynamik. Die Untersuchung der Wechselwirkungen zwischen Austern und *Vibrio spp.* ist wichtig, um bakterielle Erkrankungen und Sterblichkeit in der Aquakultur zu verhindern. Die Fluoreszenz-in-situ-Hybridisierung (FISH)

wurde verwendet, um die Vielfalt von *Vibrio* Kanaloae in verschiedenen Geweben sichtbar zu machen.

In Kapitel III wurden *C. gigas*-Austern von der Insel Sylt untersucht, die im Sommer 2020 den Stämmen T02 und S12 von *V. kanaloae* ausgesetzt waren. Daraufhin wurden verschiedene Austernorgane mit der kulturunabhängigen molekularen Technik der Fluoreszenz-in-situ-Hybridisierung (FISH) analysiert, um die Vielfalt von *V. kanaloae* in verschiedenen Geweben der Auster am dritten Tag nach der Infektion schnell sichtbar zu machen, da die zellulären Immunparameter am dritten Tag ihren Höhepunkt erreichten, was in erster Linie eine Reaktion auf die Immunherausforderung zeigt. Folglich bieten unsere Ergebnisse eine neue Perspektive auf die Wirksamkeit der Invasion. Bei einer Untersuchung der In-vivo-Verteilung wurde festgestellt, dass die Verdauungsorgane, Kiemen und Muskeln *Vibrio kanaloae* enthalten.

Die Aquakulturproduktion (Fische, Austern, Weichtiere) überstieg 2019 ,177,8 Millionen Tonnen. *Vibrio splendidus* beeinträchtigt die weltweite Produktion von *C. gigas*-Austern. Austern verfügen über ein integriertes, hochkomplexes angeborenes Immunsystem zur Erkennung und Beseitigung von Eindringlingen, das sowohl eine angeborene als auch eine adaptive Immunität aufweist. Das Genom der Pazifischen Auster *C. Gigas* hat eine massive Ausbreitung und funktionelle Divergenz von Genen des angeborenen Immunsystems gezeigt. Immunpriming verstärkt die Immunreaktion bei einer späteren Exposition gegenüber *V. splendidus*. Immunpriming umfasst zwei mechanistische Modelle: eine biphasische Reaktion, gefolgt von einer Extinktionsphase, und eine einmalige Reaktion, die nach dem Priming ausgelöst wird. Immunstimulanzien können die Widerstandskraft von Wasserorganismen gegen Infektionskrankheiten erhöhen, indem sie ihre Abwehrmechanismen verstärken.

In Kapitel IV untersuchte ich, ob Pazifische Austern (*Crassostrea gigas*), die mit 10^4 *Vibrio splendidus* geprimt wurden, eine stärkere Immunantwort zeigten, wenn sie mit 10^7 *V. splendidus* herausgefordert wurden. Außerdem inokulierten wir die Austern gegen *Vibrio*, indem wir sie sympatrischen und allopatrischen *V. splendidus*-Stämmen aussetzten, um ihre Resistenz in vivo zu bewerten und festzustellen, ob diese Besiedlung die Virulenz und das Überleben erhöht. Austern, die gegen den sympatrischen Stamm von *V. splendidus* geschützt waren, überlebten eine zweite Herausforderung besser. Darüber hinaus untersuchten wir das Priming des Bakteriums *Vibrio* durch die Exposition gegenüber kolonisierten Austern, um ihre Resistenz gegen Hämolymphe in

vivo zu bestimmen. Wir analysierten die immunologischen Reaktionen der Austern, um festzustellen, ob die Besiedlung zu einer Erhöhung der Sterblichkeit führt. Im Austernmodell zeigten die ausgewählten Isolate eine erhöhte Überlebensrate sowohl bei der primären als auch bei der sekundären Herausforderung sowie einen In-vitro-Schutz gegen *V. splendidus*. Selektive Austernpopulationen, die gegen *V. splendidus* resistent sind, zeigten eine erhöhte Überlebensrate in vivo, eine hemmende Wirkung auf *V. splendidus* in vitro während einer sekundären Herausforderung und einen Kreuzschutz gegen *V. splendidus* in vitro-Untersuchungen.

In dieser Arbeit habe ich Hypothesen untersucht, die erklären, warum die Seegurke *H. leucospilota* antibakterielle Wirkungen gegen die *S. aureus*-Stämme MRSA, SEASA und SEBSA hat, aber es sind noch weitere Forschungen erforderlich. FISH ist eine wertvolle und schnelle Technik zum Nachweis und zur Quantifizierung der lebenden Formen von *V. kanaloae*. Mit der entwickelten Methode können lebensfähige *V. kanaloae* in Austern identifiziert werden. Mit dem Vib-16S-1-Gerät lässt sich die am häufigsten vorkommende pathogene *Vibrio*-Spezies bestimmen. Aufgrund der direkten Visualisierung der Bakterien kann die Nachweiszeit im Vergleich zu kulturbasierten Methoden erheblich verkürzt werden. Es konnte gezeigt werden, dass Immunopriming die humorale Immunantwort von Austern sowohl gegen sympatrische als auch gegen allopatrische Stämme von *V. splendidus* stimuliert, um das spezifische Gedächtnis und die adaptive Immunität in Austern zu verbessern. Dies könnte für orale Impfprogramme zur Verhinderung von Krankheitsausbrüchen in der Aquakultur genutzt werden. Weitere Untersuchungen sind erforderlich, um den voraussichtlichen Mechanismus zu ermitteln, der für die beobachteten Veränderungen verantwortlich ist.

SUMMARY

Antibacterial activity of marine sea cucumber and oyster against *Staphylococcus aureus and Vibrio*

Oceans and seas are home to a large proportion of all life on Earth, with 71% of the Earth's surface being ocean water, while only 2.5% is drinkable freshwater. Oceanic plankton, algae, and certain bacteria contribute significantly to this large proportion. Marine habitats are divided into coastal and open ocean habitats, with coastal habitats occupying only seven per cent of the ocean surface. The organisms studied range from microscopic phytoplankton and zooplankton to whales in the open ocean. Invertebrates are an important part of marine life, providing food, medicine, and raw materials. Advancement of invertebrate-derived compounds is a therapeutic prospect for biomedical discovery. Marine natural product drug discovery efforts have yielded nine approved drugs and 12 currently under clinical trial, largely from collections of marine invertebrates from shallow-water tropical ecosystems. The global fish production industry has a significant impact on national economies, providing 17% of the animal protein consumed by the global population, and providing health benefits. Aquaculture is a significant contributor to the global food fishery industry.

Sea cucumber is a marine invertebrates widely consumed in East Asia due to its multiple biological and pharmacological properties, including anticancer, anti-angiogenic, anticoagulant, anti-inflammatory, anti-hypertension, antimicrobial, antithrombotic, antioxidant, and antitumor properties. The Persian Gulf is a biodiverse ecosystem with *Holothuria leucospilota* being the most commonly harvested sea cucumber. However, only 20 articles have investigated the Persian Gulf's Holothurian species. Previous investigations have shown that high concentrations of Holothurian compounds have a weak antibacterial effect. Sea cucumber protein hydrolysates are a healthy and

reliable alternative to artificial food preservatives. *Staphylococcus aureus* is one of the most prevalent pathogens responsible for food poisoning, and the emergence of antimicrobial resistance in *S. aureus* is a public health concern.

In chapter II, I studied by using extraction of bioactive compounds from sea cucumber *Holothuria leucospilota* revealed antibacterial effects on MRSA and enterotoxin-producing *Staphylococcus aureus* strains (SEASA, SEBSA) were determined. Three methods were used to determine antimicrobial activity: disc diffusion experiments, the minimum bactericidal concentration (MBC), and the minimum inhibitory concentration (MIC). The results indicate that methanol and chloroform extracts inhibit the growth of all tested isolates at MIC concentrations as high as 100 mg/ml. In concentrations of approximately 100 mg/ml, the chloroform extract exhibited bactericidal activity against SEBSA. In concentrations below 100 mg/ml, the extract is also bactericidal against MRSA and SEBSA. Methanol extract demonstrated the greatest antibacterial activity. Therefore, sea cucumber extract is a promising candidate for the discovery of new antimicrobials; however, extensive research is required to isolate and identify the active components of *Holothuria leucospilota* from the Persian Gulf. I found Sea cucumber extracts can considered to possess antimicrobial properties. My results support the idea that the sea cucumber extracts are considered a healthy and dependable alternative to artificial food preservatives is illustrative of their antimicrobial activity.

Aquaculture contributes to global fish production and health benefits.Vibriosis is a potentially fatal infection caused by *Vibrio* species, which are abundant in marine and estuarine ecosystems and can cause human cutaneous infections and gastrointestinal disorders. *Vibrio spp* is increasing due to climate change, and Pacific Oyster *Crassostrea gigas* is an appropriate model for the study of *Vibrio* and host interactions in disease dynamics. Investigation of interactions between oysters and *vibrio.spp* is essential to prevent bacterial diseases and mortality in aquaculture. Fluorescence in situ Hybridization FISH was used to visualize diversity of *v.Kanaloae* in different tissues.

In chapter III, investigated on the *C. gigas* oysters from Sylt Island were exposed10^8 *V. kanaloae* strains T02 and S12 in the summer of 2020 .Consequently, different oyster organs were analyzed by the culture-independent molecular technique, Fluorescence in situ Hybridization (FISH) to rapidly visualize the diversity of *v.Kanaloae* in different tissues of the oyster on the 3rd day post-infection due to the fact that cellular immune parameters peaked on day 3 demonstrating primarily

a response to the immune challenge. Consequently, our findings offer a novel perspective on invasion efficacy. During an investigation into its in vivo distribution, the digestive organs, gills, and muscles were found to contain *Vibrio kanaloae.*

Aquaculture production including fish, oysters, molluscs exceeded 177.8 million metric tonnes in 2019. *Vibrio splendidus* is affecting global production of *C. gigas* oysters. Oysters have an integrated, highly complex innate immune system to identify and eliminate invaders, with both innate and adaptive immunity. The genome of the Pacific oyster *C. Gigas* has revealed massive expansion and functional divergence of innate immune genes. Immune priming in enhances the immune response upon subsequent exposure to *V. splendidus*. Immune priming involves two mechanistic models: a biphasic response followed by an extinction phase, and a unique response initiated after priming . Immunostimulants can increase the resistance of aquatic organisms to infectious diseases by enhancing their defense mechanisms.

In chapter IV, I investigated on Pacific oysters (*Crassostrea gigas*) primed with 10^4 *Vibrio splendidus* exhibited stronger immune responses when challenged with 10^7 *V. splendidus*. In addition, we inoculated the oysters against *Vibrio* by exposing them to sympatric and allopatric *V. splendidus* strains in order to evaluate their resistance in vivo and determine if this colonization increases virulence and survival. Oysters that had been cross-protected against the sympatric strain of *V. splendidus* survived a secondary challenge better. In addition, we examined the priming of the bacterium *Vibrio* by exposure to colonized oysters in order to determine their resistance to haemolymph *in vivo* experiment. We analyzed the immunological responses of oysters to determine if colonization leads to an increase in mortality. In the oyster model, the selected isolates demonstrated increased survival in both the primary and secondary challenges, as well as *in vitro* protection against *V. splendidus.* Selective populations of oysters resistant to *V. splendidus* exhibited an increase in *in vivo* survival, an inhibitory effect on *V. splendidus in vitro* during a secondary challenge, and cross-protection against *V. splendidus* in vitro examination.

In this thesis, I investigated hypotheses explaining the Sea Cucumber *H. leucospilota* has antibacterial effects against *S. aureus* strains MRSA, SEASA, and SEBSA, but further research is needed. FISH is a valuable and rapid technique for detecting and quantifying *V. kanaloae's* living forms. Using the developed method, viable *V. kanaloae* in oysters can be identified. The Vib-16S-1 instrument can be used to determine the pathogenic species of *Vibrio* that is most prevalent. Due to the direct visualization of bacteria, the detection time can be significantly reduced compared to culture-based methods. Immunopriming was shown to stimulate oyster humoral immune

responses against both sympatric and allopatric strains of *V.splendidus* in order to improve specific memory and adaptive immunity in oysters. which could be used for oral vaccine programs to prevent aquaculture disease outbreaks. Further investigation is required to determine the prospective mechanism responsible for the observed changes.

Objective

In my dissertation, I conducted an in-depth investigation into microbial interactions in aquatic organisms and their implications for seafood safety, disease dynamics, and biocontrol strategies. The study delves into the intricate realm of microbial interactions, with a specific focus on the sea cucumber *Holothuria leucospilota* and the Pacific oyster *Magalana gigas.* Through a series of interconnected chapters, the research endeavors to establish a thorough understanding of these interactions, offering valuable insights into seafood safety, the dynamics of diseases, and the potential development of biocontrol strategies.

Sea cucumber *Holothuria leucospilota* Extracts

This chapter explores the antimicrobial potential of extracts obtained from *Holothuria leucospilota,* emphasizing their effectiveness against *Staphylococcus aureus*, including methicillin-resistant strains and enterotoxin producing *Staphylococcus aureus* strains (SEASA, SEBSA. The primary objective was to identify bioactive compounds within the sea cucumber extracts possessing antibacterial properties. By doing so, this chapter lays the groundwork for investigating natural antimicrobials in marine organisms, positioning the sea cucumber as a promising source for the discovery of novel antimicrobial agents and a particular emphasis on their suitability as substitutes for synthetic food preservatives.

***Vibrio* Dynamics in Oysters**

Chapter III examined the relationship between Pacific oysters (*Crassostrea gigas*) and *Vibrio* species, particularly *Vibrio kanaloae,* in the context of disease dynamics. The technique of molecular fluorescent in situ hybridization (FISH) was used to quickly determine the range of bacteria present in *Crassostrea gigas*. This allowed for important observations on the metabolic activity and distribution of *Vibrio* strains throughout the tissues of the oysters. This chapter establishes the fundamental concepts necessary to comprehend the dynamics between hosts and

pathogens, thereby creating a foundation for investigating the influence of microbial interactions on the health of oysters.

Immune Priming in Oysters

In this chapter, our main goal is to investigate the specificity of immune priming responses in Pacific oysters (*Magalana gigas*) as a potential strategy for biocontrol against pathogenic *Vibrio* strains, particularly *Vibrio splendidus*, with a focus on mitigating high summer mortalities in aquaculture. We explore the nuances of immune priming by exposing oysters to sympatric and allopatric *V. splendidus* strains, assessing the inhibitory effects of haemolymph plasma, and examining cross-protection against lethal *Vibrio* challenges. The research sheds light on the correlation between strain origin and the effectiveness of immune priming, emphasizing the potential of matching local strains for optimal protection. Ultimately, the findings aim to contribute to the development of targeted biocontrol measures to safeguard oyster production and address economic implications in the food industry.

Conclusion

Expanding on the knowledge acquired from Chapters II, III, and Chapter IV, this section explores the immunological priming responses demonstrated by Pacific oysters, namely *Magalana gigas*, when exposed to pathogenic *Vibrio* strains. The objective is to examine the degree of immunological priming specificity by studying closely similar strains of *Vibrio splendidus.* This chapter attempts to investigate the possibility of immune priming as a biocontrol technique against high summer mortalities in oyster populations by analyzing the inhibitory effects of oyster haemolymph on *Vibrio* growth.Collectively, these chapters establish a coherent story that covers the discovery of natural antimicrobials in marine organisms (Chapter II), the comprehension of disease patterns in oysters (Chapter III), and the investigation of immune priming as a possible approach to control *Vibrio*-induced mortality in aquaculture (Chapter IV). This dissertation enhances our comprehension of microbial interactions in aquatic habitats, which has significant implications for ensuring seafood safety and implementing sustainable management practices in aquaculture systems.

CHAPTER I: GENERAL INTRODUCTION

Oceans and seas are home to a large proportion of all life on Earth (Sala *et al.*, 2021). Due to the fact that many marine species are still unknown, it is difficult to determine the exact size of this large proportion. Oceans cover approximately 71% of the surface of the Earth and are a complex three-dimensional world (Sala *et al.*, 2021). The majority of the water on our planet is ocean water, while only 2.5% is drinkable freshwater. The oceans produce roughly half the oxygen on the planet (Jm *et al.*, 2018). Oceanic plankton, algae, and certain bacteria capable of photosynthesizing contribute most of this production.

There are two main types of marine habitats: coastal habitats and open ocean habitats (Hopf *et al.*, 2016). An area of coastal habitat extends from the shoreline to the edge of the continental shelf. The majority of marine life is found in coastal habitats, although the area occupies only seven per cent of the total ocean surface (Walters, Hilborn and Parrish, 2007)). A marine habitat may be modified by its inhabitants. Coral, kelp, and sea grass are some marine organisms that are ecosystem engineers, reshaping the marine environment in order to provide new habitats for other species (Walters, Hilborn and Parrish, 2007).

The ocean is structured into estuaries, coral reefs, kelp forests, seagrass meadows, seamounts and thermal vents, tidepools, sandy, muddy and rocky bottoms, and the open ocean (pelagic zone), where solid objects are rare and the surface of the water is the only boundary that is visible (Walters, Hilborn and Parrish, 2007).The "sandy," "muddy," and "rocky" bottoms describe different seafloor compositions in marine environments, including intertidal zones. These characteristics denote the predominant substrate types, with sandy bottoms associated with moderate water movement, muddy bottoms in calmer waters, and rocky bottoms providing hard substrate for marine organisms (Walters, Hilborn and Parrish, 2007) The organisms studied range from microscopic phytoplankton and zooplankton to whales that are 25 to 32 meters in length.

In addition, it is becoming increasingly apparent that marine organisms and other organisms are fundamentally interconnected. It is estimated that the body of knowledge regarding the relationship between life in the sea and important cycles is rapidly expanding, with new discoveries being made

nearly every day(Sala *et al.*, 2021) and (Jm *et al.*, 2018). In addition to the carbon cycle, these cycles involve those of matter (such as Earth's respiration) and air (such as energy movement through ecosystems, including the oceans). It is estimated that there are large areas beneath the ocean's surface that are virtually unexplored.

Similarly, to land, invertebrates account for a large portion of all marine life (Guénette and Pitcher, 1999). Invertebrate species in the sea include Cnidaria, such as jellyfish and sea anemones, Ctenophora, sea worms, such as Platyhelminthes, Nemertea, and Phoronida, and Mollusca, such as shellfish, squid, and octopus, Arthropoda, such as Chelicerata and Crustacea, Porifera and Bryozoa, Echinodermata, including starfish, sea cucumbers, and Urochordata, including sea squirts and tunicates (Guénette and Pitcher, 1999) .The marine environment is a vast resource, providing food, medicine, and raw materials. Fish and shellfish are important economic resources to humans (Blunt *et al.*, 2016 ; Radjasa *et al.*, 2011). In 2019, the appropriate systems for producing edible protein aquaculture, including fish, oysters, molluscs, crustaceans, and other aquatic species for human consumption, surpassed 177,8 million metric tonnes, up from 147.2 million metric tonnes in 2010 (Radjasa *et al.*, 2011 ; Gerwick and Fenner, 2013) .The advancement of invertebrate-derived compounds is designated as a therapeutic prospect for biomedical discovery. The focus is on secondary metabolites, their structural inspirations, and the potential function of microorganisms in their biosynthesis (Radjasa *et al.*, 2011) .

In recent years, the marine environment has been a source of more than 20,000 natural products have been discovered (Gerwick and Fenner, 2013) . As a result of these efforts, nine approved drugs and 12 currently under clinical trial have been discovered, either as natural products or as molecules inspired by natural products (Gerwick and Fenner, 2013). As an example, Omega-3 fatty acids, specifically eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are abundant in fish. Omega-3 fatty acids play a vital role in numerous facets of human health, encompassing but not limited to cardiovascular health, cognitive vitality, and inflammation reduction (Aucoin *et al.*, 2017). For the treatment of severe and chronic pain, ziconotide, an analgesic derived from a peptide discovered in the secretion of the cone snail, is prescribed (Molinski *et al.*, 2009). Ecteinascidin 743 (Yondelis) ,Originally isolated from the Caribbean sea squirt *Ecteinascidia turbinata*,(Manning *et al.*, 2006)Yondelis is an antitumor drug used in the treatment of soft tissue sarcoma and ovarian cancer.Cytarabine (Cytosar-U) ,Although it is now

produced synthetically, cytarabine, an anticancer drug used in the treatment of leukemia, was originally isolated from a Caribbean sponge called *Tethya crypta* (Manning *et al.*, 2005; Paniagua-Michel *et al.*, 2015). Bryostatin 1: Originally isolated from the marine *bryozoan Bugula* neritina, bryostatin 1 has shown promise in cancer treatment and has potential applications in the treatment of Alzheimer's disease (Manning *et al.*, 2005).

This pharmaceutical substance serves as an illustration of the ability of marine organisms to produce therapeutic compounds, thus emphasizing the ongoing captivation with biomedical research.To a significant degree, these have come from collections of marine invertebrates largely obtained from shallow-water tropical ecosystems. In addition, due to the inherent multidisciplinary nature of this discipline, marine natural product drug discovery efforts are characterized by a high level of innovation (Gerwick and Fenner, 2013 ; Hossain *et al.*, 2022) .

The sea cucumber is a marine invertebrates and member of the class *Holothuroidea* and the phylum Echinodermata; it is found worldwide in deep seas and benthic environments (Hossain *et al.*, 2020) . It is extensively consumed in China, Korea, Japan, Malaysia, Indonesia, Iran and Russia. It has a leathery exterior, a soft, cylindrical body, and a gonad with a solitary branch(Wen, J, *et al.*, 2010) .xtremely limited in fat and cholesterol, sea cucumbers are rich in protein (Wen, J, *et al.*, 2010) . There are approximately 1500 species of sea cucumbers in the world (Eckert, G.L ,2007), and about 100 of them are commonly consumed (Purcell, S.W, *et al.*, 2007). The most important commercial species are *Apostichopus japonicus, Acaudina molpadioides, Actinopyga mauritiana, Cucumaria frondosa, Cucumaria japonica, Holothuria forskali, Holothuria polii, Holothuria nobilis, Holothuria tubulosa, Isostichopus badionotus,* and *Pearsonothuria graeffei* (Hossain *et al.*, 2020) . Hossain demonstrated due to its potential therapeutic benefits and as a marine dietary item, sea cucumber has received increased attention. Furthermore, owing to the availability of its biologically active compounds with medicinal properties, it has gained increasing interest as a functional food ingredient. (Hossain *et al.*, 2020) . They contain bioactive compounds such as phenolics, polysaccharides, proteins, carotenoids, and saponins, which have antioxidant properties . (Hossain *et al.*, 2022) .Sea cucumbers have been shown by Wen J. (2010) to be higher in protein and lower in fat, The predominant amino acid was lysine, and the lysine-to-arginine ratio was low across all species. More monounsaturated and polyunsaturated fatty acids were present in *A. mauritiana* (Wen, J, *et al.*, 2010) .The proximate composition and fatty acid content of two species

of sea cucumber, *Holothuria scabra* and *Holothuria edulis*, are compared by Chen, J. (2003) . In the coastal waters of Sabah, *H. edulis*, a low-valued noncommercial species, is prevalent, whereas *H. scabra* is highly valued. The research revealed that *H. edulis* possesses a greater quantity of protein than *H. scabra*, indicating that it might serve as a viable substitute for *H. scabra* species in the nutraceutical industry and alleviate strain on the overly exploited species. (Chen, J. 2003).

Triterpene glycosides, secondary metabolites of sea cucumbers, have various biological activities, including hemolytic, cytotoxic, and antifungal properties(Aminin DL . 2015). They suppress human tumor cell proliferation in vitro and significantly reduce tumor burden and metastasis in rodents. These glycosides induce tumor cell apoptosis, arrest cell cycles, and regulate cellular receptors and enzymes involved in cancerogenesis. They reduce cancer cell adhesion, suppress migration, angiogenesis, and cell proliferation, leading to significant tumor growth inhibition. (Aminin DL . 2015).

The chemical compounds isolated from various sea cucumbers exhibit unique biological and pharmacological properties, including anticancer ,(Aminin, D.L, 2015 ; Janakiram, N.B , *et al.*, 2015) anti-angiogenic (Tian, F, *et al.*, 2018) anticoagulant (Nagase, B.H ,*et al.*, 1995 ; C hen, S,*et al.*, 2011) anti-inflammatory (Borsig, L, *et al.*, 2007) anti-hypertension (Kiew, P.L, *et al.*, 2012) antimicrobial (Beauregard, K.A, *et al.*, 2001; Hing, H, *et al.*, 2007) antithrombotic (Mourão, P.A.S, *et al.*, 2001) and antitumor (Althunibat, O.Y *et al.*, 2009) . In addition, bioactive components derived from sea cucumbers can be applied to the mouth, face,nhair, joints, and cranium as novel cosmetic ingredients (Siahaan, E *et al.*, 2017) . Malaysians use extracts of sea cucumber skin to treat hypertension, asthma, wound healing, scrapes, and burns (Taiyeb-Ali, T.B, *et al.*, 2003).Sea cucumber has a remarkable nutritional profile, including protein (primarily collagen), lipid (primarily omega-3 and omega-6 fatty acids), and carbohydrates. Extract of sea cucumber is able to boost innate immune responses and inhibit intestinal tumors in rodents (Janakiram NB, *et al.*, 2015). The activity of these bioactive compounds is dependent on the species, harvesting location, dietary habits, body segment, and processing techniques (Hossain A,*et al.*, 2020). Sea cucumbers play an important role in the recycling and remineralization of organic matter (OM) in reef sands through feeding, excretion, and bioturbation (the biogenic mixing of sediment)processes(Lee S, *et al.*, 2018). As a result of decreasing marine pollution, sea

cucumbers may be able to reconcile the incompatibility between fish production and environmental conservation.

Chapter II

The Persian Gulf (Iran's coastal waters) is a unique, biodiverse ecosystem (Ghadiri, M.,*et al.,* 2018*;* Mashjoor S *et al*., 2019) (Figure 1). *Holothuria leucospilota*, also known as the black tarzan or black sea cucumber, has been the most commonly harvested sea cucumber in the Persian Gulf since 2004 at Qeshm Island(Afkhami,M *et al.,* 2012) (Figure 2). *H. leucospilota* produces bioactive secondary metabolites, making it an attractive organism for drug discovery (Bordbar, S, et al., 2011) Due to the growing demand for sea food and drug candidates, the study of sea cucumbers has increased (Aydın, M, *et al.,* 2011*;* Pawson, D. L *et al.,* 2007*).* However, of the nearly 1400 scientific publications recorded worldwide about Holothurian species (Ghadiri, M.,*et al.,* 2018*;* Mashjoor S *et al*., 2019) , only about 20 investigated the Persian Gulf's Holothurian species. Therefore, the biological and antimicrobial activity of sea cucumber populations in this region is understudied (Mokhlesi, A, et al., 2011) Previous investigations have demonstrated that very high concentrations of Holothurian compounds have a weak antibacterial effect (Rinehart, K. L.,et al.,1981; Bryan, P ,J et al., 1994).When tested against human pathogens, extracts from various organs or tissues of the sea cucumber *Holothuria* exhibited limited bacteriostatic activity eluded with methanol, chloroform, or n-hexane(Afkhami,M *et al.,* 2012). Compared to conventional antimicrobials, the efficacy of purified Holothurian peptides as antibacterial agents was relatively low (Tian, F, *et al*., 2005). Antimicrobial activity has been proposed as sea cucumber protein hydrolysates are considered a healthy and reliable alternative to artificial food preservatives (47,48) (Borsig, L, *et al.,* 2007). (Mamelona, J., *et al.,* 2018; Gaidi, G *et al.,* 2001)

Heat-stable staphylococcal enterotoxins (SEs) produced by enterotoxigenic strains of *Staphylococcus aureus* (Schlievert, P. M. *et al.,*1981;LeLoir, Y., *et al.,* 2003) are a significant global cause of food poisoning. *S. aureus* is regarded as one of the most prevalent pathogens responsible for food poisoning outbreaks (*Normanno, G et al.,* 2005 ; *Vasconcelos, N. G. et al.,*2010). The pervasive use of antibiotics has resulted in the emergence of multidrug-resistant strains, making it more difficult to eradicate the diseases they cause and increasing their prevalence (Hammad, A. M *et al.,* 2012). The rising prevalence of antimicrobial-resistant *S. aureus* is a significant threat to food safety and healthcare systems (*Argudína, M. A. et al.,* 2012).In particular,

the emergence of antimicrobial resistance in *S. aureus* is a significant public health concern..MRSA has been detected in aquatic animals(58),and the number of resistant MRSA isolates is increasing (Rhee, C. H. *et al.*,2010; Argudína, M. A.., *et al*., 2012) These investigations have raised additional food safety concerns about *S. aureus* beyond its role as a food-poisoning agent (Pu, S., Wang, F. & Ge, B 2010; Arfatahery, N;. *et al.*,2016).

Preformed *Staphylococcus aureus* enterotoxins are one of the most prevalent causes of seafood-borne food poisoning around the globe(Arfatahery, N;.*et al.*,2016)). Aquatic organisms, including those consumed as seafood, are also a source of potentially important organic compounds. Bioactive compounds of the sea cucumber *Holothuria leucospilota* were extracted using chloroform or methanol as part of an investigation into the antimicrobial activity of marine macro organisms from the Persian Gulf. The antibacterial effects of the extracts on methicillin-resistant *Staphylococcus aureus* (MRSA) and enterotoxin-producing *Staphylococcus aureus* strains (SEASA, SEBSA) were determined.

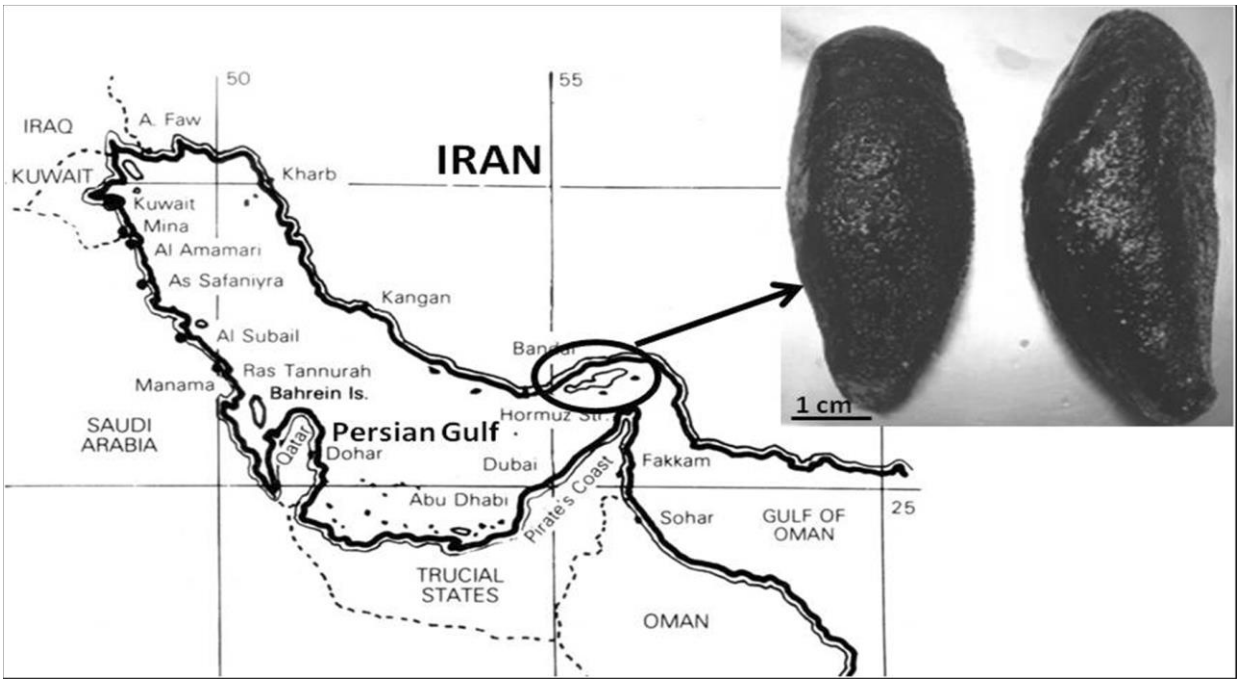

Figure1: Map of the Persian Golf indicating the sampling area and the sea cucumber collected for the study(Ghadiri, M , 2018)

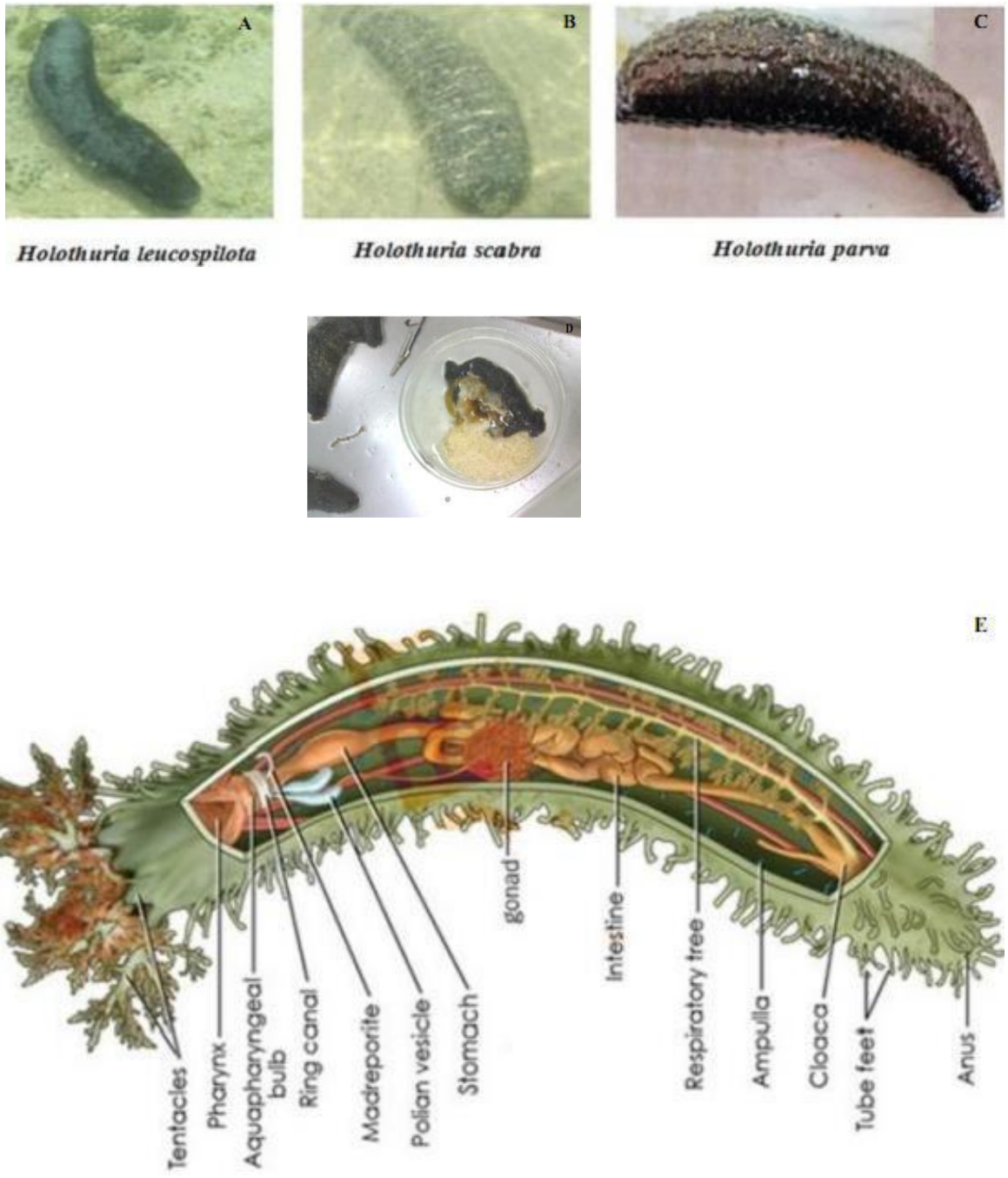

Figure2: Mashjoor's study(2019), (labeled A-D) the intricate internal organs of three distinct marine *Holothuria* species from the Persian Gulf are meticulously dissected .(E)The detailed anatomical features revealed in this study contribute to a comprehensive understanding of *Holothuria* sea cucumbers (Mashjoor S;.*et al.*,2019).

Objectives Chapter II

Objectives Due to the production of distinctive naturally occurring bioactive compounds, research into aquaculture drug sources such as the sea cucumber *H. leucospilota* is necessary. The medicinal and antibacterial effects of sea cucumbers are well-known. In chapter II of this thesis, I investigated the hypothesis that the antibacterial activities of methanol compared to chloroform extracts from the whole body of *H. leucospilota* against methicillin-resistant *Staphylococcus aureus* (MRSA) and enterotoxin producing *Staphylococcus aureus* strains (SEASA, SEBSA). Minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) were utilized to determine antimicrobial activity. At MIC concentrations up to 100 mg/ml, methanol and chloroform extracts inhibit the growth of all strains tested. In approximately 100 mg/ml concentrations, the chloroform extract demonstrated bactericidal activity against SEBSA. The extract at concentrations below 100 mg/ml is also bactericidal against MRSA and SEBSA. The methanol extract exhibited the greatest antibacterial activity. The study specifically aims to gauge the potential of sea cucumber extracts, recognized for their unique naturally occurring bioactive compounds, as viable alternatives for antibiotics and anti-contamination agents. The acquired results are anticipated to enhance our comprehension of the antibacterial attributes inherent in sea cucumber extracts, with a particular emphasis on their suitability as substitutes for synthetic food preservatives. Furthermore, the investigation endeavors to underscore the necessity for further research aimed at isolating and identifying the active compounds responsible for the observed antibacterial efficacy in *Holothuria leucospilota* sourced from the Persian Gulf.

Chapter III

The global seafood production industry has a significant impact on national economies, with net exports from developing countries worth $38 billion in 2018 (FAO (2020). Fish and sea foods serve a vital role in global food security, providing 17% of the animal protein consumed by the global population in 2017(FAO (2020). Seafood consumption is associated with health benefits, such as a reduced risk of death from coronary heart disease. Many nations have incorporated seafood consumption recommendations into their national dietary guidelines, but few incorporate environmental considerations. This is crucial due to the expanding constraints on the global seafood supply (FAO and WHO (2010) .Aquaculture is a significant contributor to the global food

fishery industry, with 177.8 million metric tonnes of edible fish produced in 2019 (FAO (2020). Vibriosis is recognized as a significant factor impeding the development of aquaculture (Figure3). Vibriosis is a potentially fatal infection induced by Vibrio species (Chen, F.R.;.et al.,2004 ;Thompson, F.L;et al.,2004). Vibrios are gram-negative bacteria that are abundant in marine and estuarine ecosystems and can be found free-living in the water column, as part of a biofilm, or associated with a host (Austin, B.,and D.A. Austin. 2007;Thompson, F.L;*et al*.,2004)(Figure4).Some of the rapidly expanding number of Vibrio species that can cause human cutaneous infections and gastrointestinal disorders have been linked to human disease(Venkateswaran, K.,.;.*et al*.,1998 ; Andrews, L.S;*et al*.,2004).

Vibrio splendidus, a pathogen associated with summer mortalities, has a global impact on the production of *Crassostrea gigas* oysters(Andrews, L.S. 2004,*et al*.,2004). The bivalve species *Crassostrea gigas*, more commonly referred to as the Pacific oyster, is a commercially significant organism distinguished by its adaptability and swift growth. Originating in the Pacific coast of Asia, this species has since been introduced and cultivated on a global scale due to its significant economic value within the aquaculture sector. (Wendling Carolin C. and Wegner K. Mathias 2015) *Vibrio. spp* can be found in a wide variety of habitats in aquatic and marine environments (Brumfield KD.;. *et al.*,2021).

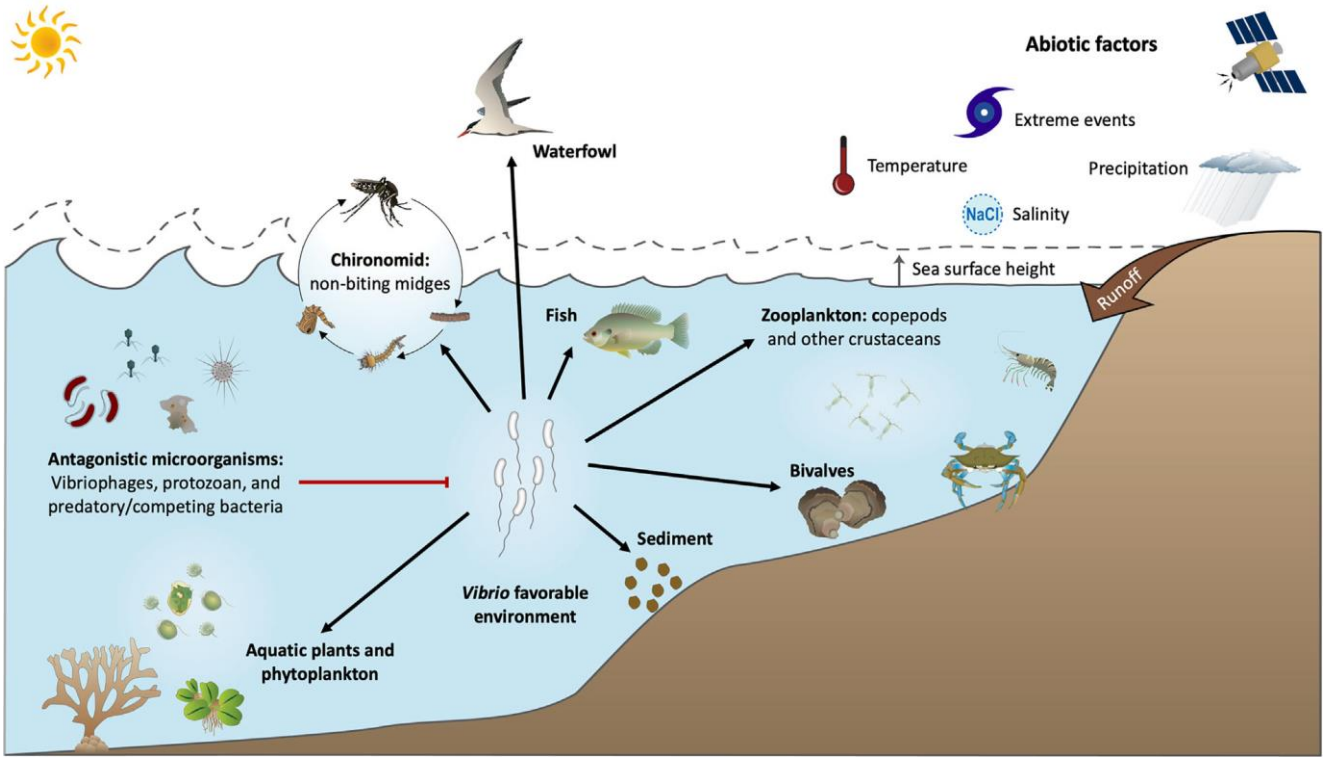

Figure3: Adapted from Sakib *et all* (2018) study on interactions between Vibrio spp. and their natural environment.Presented are the biotic and abiotic factors that influence the incidence and distribution of Vibrio spp. in their natural habitat. Black arrows represent potential reservoirs, including zooplankton (primarily copepods), crustaceans, bivalves, fish, aquatic plants, phytoplankton, chironomids, waterfowl, and detritus; red arrows represent environmental Vibrio spp. antagonists, including Vibrio phages, protozoa, and predatory/competing bacteria. Abiotic factors, such as temperature, sea surface height, precipitation, and extreme weather events, are capable of influencing the size and metabolic activity of Vibrio spp. populations. To protect public health, it is essential to ascertain the effect of abiotic factors on the occurrence and distribution of pathogenic Vibrio spp. Using the University of Maryland Centre for Environmental Science Integration and Application Network Image Library, an image was created (UMCES, 2021).

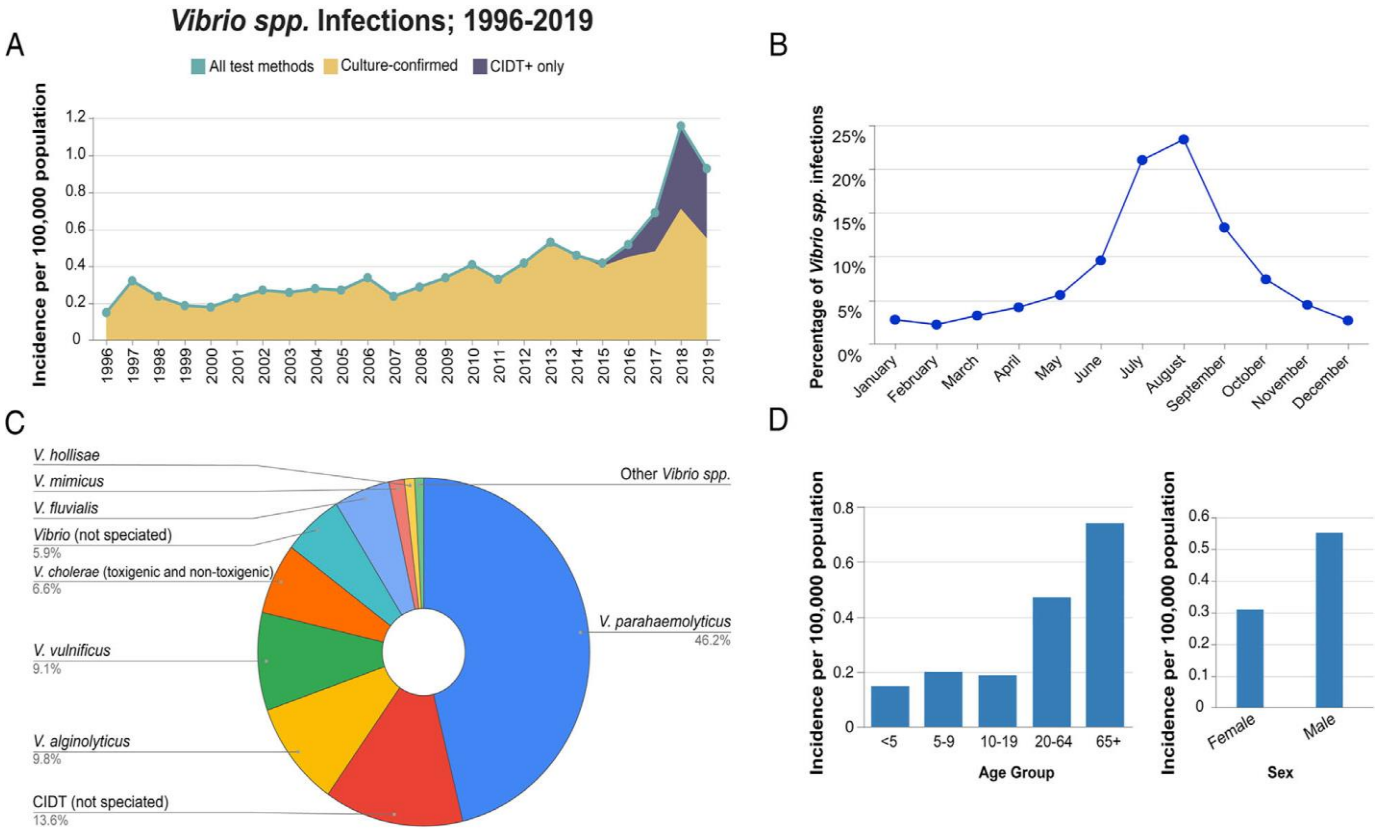

Figure 4:Pathogen surveillance of infections attributable to pathogenic *Vibrio spp*., 1999–2019 (CDC, 2021). Using the Foodborne Diseases Active Surveillance Network (Food Net) Fast, graphs for Vibrio infections were generated. Where indicated, data are presented as the number of infections per 100,000 population at Food Net sites, which comprise 10 states and approximately 15% of the population of the United States.**A**. Infections with *Vibrio* by year. The incidence of infections caused by pathogenic *Vibrio spp.* is displayed. Teal, all test methods; gold, culture confirmed, including infections confirmed by culture alone or by culture after a positive culture-independent diagnostic test (CIDT); and purple, CIDT only.**B**. The monthly percentage of infections among all reported cases is displayed.**C**. Infections brought on by pathogenic *Vibrio spp.* are represented as a proportion of all reported cases. CIDT, culture-independent diagnostic test.**D**. Population characteristics of infections caused by pathogenic *Vibrio spp*.The average annual incidence of infections by age (left) and gender (right) is depicted (CDC, 2021).

GENERAL INTRODUCTION

Vibrio isolation from decomposing and healthy *C. gigas* has been conducted for more than three decades (Tubiash HS;*et al*.1970 ; Sugumar G,;.*et al*.,1998; Waechter M; *et al*.,2002 ;Estes RM; *et al*.,2004; Wegner KM;*et al*.,2014). Current investigations indicate that *V. tubiashii, V. anguillarum, V. splendidus,* and *V. aestuarianus* are the predominant species responsible for oyster mortality (Tubiash HS;*et al*.1970). In recent years, 16S rRNA/rDNA-based PCR (polymerase chain reaction) and FISH (fluorescence in situ hybridization) have been used to detect, identify, and quantify bacterial communities in various specimens (Arellano, C.F. and Olmos, S.J. 2002). Most studies investigating microorganisms in oysters have focused on cultivable bacteria, whereas marine bacteria are typically incapable of cultivation (Colwell, R.R. and Liston, J. 1960 Ward, D.M., Weller, R. and Bateson, M.M. 1990). Few reports utilising molecular techniques describe the diversity of bacteria in apparently healthy oysters (Pujalte, M.J. 1999;Herna´ndez-Za´rate, G. and Olmos-Soto,2003; Romero, J.;*et al*.,2002). Nonetheless, substantial progress has been made in identifying the pathogenic *Vibrio* species.

In order to ascertain the pathogenicity of the local strains at high temperatures, research was conducted during the summer and experimental tests were conducted on oysters. (Wendling CC,Wegner KM ,2014). The diversity of bacteria, as well as the pathological and physiological characteristics of these bivalves, necessitate additional research. For the prevention of bacterial diseases and mortality in aquaculture (Olmos-Soto 2005), it is essential to investigate the interactions between oysters and *vibrio.spp*.

In chapters III of this thesis, I conducted on hypothesis, that one of the useful and fast tools for detecting and quantifying living forms of *V. kanaloae* is the FISH method. Identification of viable *V. kanaloae* in oysters is possible using the developed method.An ideal model for studying *Vibrio* and host and their interaction in disease dynamics is *Crassostrea gigas* in the North Sea, exposing invasive species to pathogens, especially *Vibrio kanaloae.* The oyster *C. gigas* from Sylt Island was exposed to *V. kanaloae* strains allopatricT02 and sympatric S12. Subsequently, different oyster organs were analyzed by the culture-independent molecular method Fluorescence in situ Hybridization (FISH) to rapidly visualize the diversity of bacteria in different tissues of the oyster.

Here, I studied the pacific Oyster *Crassostrea gigas* in the North Sea on the island of Sylt. This population has been studied as a model for *Vibrio* and host interactions and disease dynamics (Wendling CC, Wegner KM ,2014 ;Wegner K. Mathias 2015).In the summer of 2020, 10^8 *V.*

kanaloae strains allopatric T02 and sympatric S12 were administered to Pacific oysters from Sylt Island. Following this, the culture-independent molecular technique Fluorescence in situ Hybridization (FISH) was utilized to rapidly visualise the diversity of *V.Kanaloae* in various tissues of the oyster. The primary focus of this study was the in vivo dissemination of *V. kanaloe*, which was discovered in the digestive glands, gills, and muscles. One of the benefits of the FISH technique is its precision. This method permits rapid detection of *Vibrio* in oysters. Furthermore, Current FISH techniques designed for detection and enumeration have proved useful for predictive modelling, including the prediction of favorable environmental conditions for *Vibrio* species in oysters, as discussed in this study.

Objective Chapter III

Chapter III focuses on establishing the oyster *Crassostrea gigas* as an ideal model for studying *Vibrio* species dynamics in the North Sea. The research explores disease dynamics, particularly with *Vibrio kanaloae*, a potential pathogen. Molecular techniques, including fluorescent in situ hybridization (FISH) and 16SrRNA gene sequencing, are employed to rapidly identify and validate bacterial diversity within oyster tissues. The study examines the response of oysters to *Vibrio kanaloae* exposure, emphasizing metabolically active populations in various tissues. The research aims to understand the temporal distribution of *Vibrio* strains, especially in the gill and digestive glands. FISH proves to be a rapid and accurate method for assessing *Vibrio* abundance, with a focus on distinguishing between strains. Rigorous experimental controls address probe specificity challenges. Overall, the study contributes valuable insights into *Vibrio* dynamics in oysters, providing a foundation for future ecological and health-related investigations.

The underlying theme connecting the two chapters is the investigation of aquatic animals (specifically sea cucumber and oyster) for their potential therapeutic attributes and their interactions with bacteria, especially those that are relevant to human well-being. Both chapters enhance comprehension of the antibacterial capabilities of marine animals and their ecological functions in aquatic environments.

chapter IV

The oyster pathogen *Vibrio splendidus* is associated with elevated summer mortalities affecting the global production of *Crassostrea gigas* oysters(Pirofski, L., and Casadevall, A. ,2012). There

is growing experimental evidence of immune priming in invertebrates(Figure 5), in which prior exposure to a low pathogen burden enhances the immune response upon subsequent exposure (Yue,F*et al.*,2013; Song,X.,;*et al*.,2016) However, the underlying mechanism is not entirely understood**.**

Oysters, like other invertebrates, have evolved an integrated, highly complex innate immune system in order to identify and eliminate various invaders via a variety of orchestrated immune responses (,Juachim Kurtze ;*et al*.,2016). They have developed both innate immunity (non-specific) and there appears to be a presence of memory in the immune systems of invertebrates to combat invading foreign particles (He, X., Zhang;*et al*.,2011). Sam Locker (Adema CM;*et al* 2010) demonstrated by utilized a 70-mer oligonucleotide-based microarray to examine the transcriptomic reactions of the mollusk *Biomphalaria glabrata* in response to various immune challenges. The array comprises unidentified *B. glabrata* ESTs and non-immune factors to facilitate the discovery of functional genes. Twelve hours after wound closure, infection with compatible trematode parasites, or exposure to Gram-negative or Gram-positive bacteria, transcription profiles were documented. *B. glabrata* detects and reacts differently to compatible trematodes, with *Echinostoma paraensei* infection resulting in the downregulation of immune transcripts and *S. mansoni* exposure leading to the upregulation of features, according to the data.

It has always been thought that invertebrates, which lack lymphocytes and immunoglobulin, have only an innate immune system (Kurtz, J; *et al.*,2003).These reactions include immune recognition, the synthesis of antimicrobial peptides, signal transduction, encapsulation and phagocytosis of circulating haemocytes (He, X.,.;*et al*.,2011;Pirofski, L; *et al* ,2012). The majority of the hematopoietic tissue, hematopoiesis, and circulating haemocytes have been characterized, and the detailed annotation of the genome of the Pacific oyster *Crassostrea Gigas* has disclosed massive expansion and functional divergence of innate immune genes in this animal (Kurtz, J; *et al.*,2003).

As demonstrated by experimental evidence (Little,T.J..;*et al*.,2003, Yue, F.;*et al*.,2013, Song, X.;*et al*.,2016) and molecular evolutionary analysis (Rolff, J., Siva-Jothy, M., 2003;Ottaviani, E., 2011; Ziauddin, J., Schneider, D.S., 2012). the immune response of invertebrates may manifest adaptive characteristics, known as immune priming. However, the mechanisms underlying the observed immunological priming that affords pathogen-specific protection remain poorly understood. Antimicrobial peptides have been demonstrated to be involved by apoptosis and autophagy.

Moreover, immune priming and maternal immune transfer in oysters demonstrate the adaptability of invertebrate immunity (Kurtz, J., Franz, K., 2003.; Song, X.,, J.;*et al*.,2016).

Moreover, immune priming and maternal immune transfer in oysters illustrate the adaptability of invertebrate immunity (Kurtz, J., Franz, K., 2003.; Song, X.,, J.;*et al*.,2016).The embryos and larvae of oysters possess extraordinary antibacterial and agglutination properties against pathogens. Variations in antioxidant and hydrolytic enzymatic activities are observed during the development of the Pacific oyster *C. gigas*. Trans-generational immune priming (TGIP) refers to the increased immunity of offspring to pathogens to which their parents were exposed, and is considered an additional form of immune priming across generations. Evidence of TGIP has been found in invertebrates, such as oysters (Yue, F J.;*et al*.,2013). Various mechanistic models (Lafont M.; *et al.*,2020). have been developed to describe the implementation of immune priming. (i) a biphasic response characterized by an initial response to priming followed by an extinction phase followed by either a similar, but faster and stronger secondary response to infection (called recall response) or a qualitatively distinct secondary response (called immune shift); and (ii) a unique response that is initiated after priming, is not accompanied by an extinction phase, and is maintained throughout secondary infection (Green, Timothy J., and Peter Speck. 2018 ; Zhang, T.;*et al*.,2014).

Figure 5,Sea cucumber *Americanos Californicus* and *Crassostrea gigas* oysters prime with *vibrio. Aestuarianus.* Timothy James Green Laboratory, Vancouver Island university, North west coast Pacific Ocean 2023.Photo by Noushin Arfatahery.

Conclusion

The preceding chapters lay a foundation for a comprehensive exploration into the interplay between aquaculture drug sources, microbial dynamics, and immune responses in aquatic organisms. Chapter II initiates this journey by emphasizing the necessity of researching sea cucumber *H. leucospilota*, a prolific source of bioactive compounds known for their medicinal and antibacterial effects. The investigation into antibacterial activities against MRSA and enterotoxin-producing *Staphylococcus aureus* strains sets the stage for a deeper understanding of potential therapeutic applications. The call for future research on the chemical composition and action mechanisms acts as a segue into the subsequent chapters.

Chapter III seamlessly continues this narrative by introducing the FISH method as a valuable technique for detecting and quantifying *V. kanaloae* in oysters. The application of this method to study *Crassostrea gigas* in the North Sea as an ideal model for *Vibrio*-host interactions expands the scope from antimicrobial properties to microbial dynamics within a model organism. The exposure of oysters to *V. kanaloae* strains and subsequent FISH analysis provides insights into bacterial diversity across different oyster organs.

As the exploration deepens in Chapter IV, the focus shifts to immune responses in Pacific oysters following inoculation with *V. splendidus*. This chapter bridges the gap between microbial interactions and host immune systems, investigating the resistance of *V. splendidus* strains to innate immunity and the potential for immune priming in oysters. The monitoring of immune priming and cross-protection levels adds a layer of complexity, contributing to the understanding of how aquatic organisms respond to primary and secondary bacterial exposures.

Together, these chapters form a cohesive narrative, progressing from the identification of bioactive compounds to the application of advanced methodologies for microbial detection and, ultimately, delving into the intricate relationship between bacterial exposures and immune responses in oysters. The exploration opens avenues for potential applications, emphasizing the importance of a holistic approach in enhancing the defense mechanisms of aquatic organisms against infectious diseases.

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CHAPTER II:Antibacterial activity of the sea cucumber *Holothuria leucospilota* whole-body extract against methicillin-resistant and enterotoxin-producing *Staphylococcus aureus* strains

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Abstract

Preformed enterotoxins of *Staphylococcus aureus* are one of the most common causes of seafood-borne food poisoning worldwide. Aquatic organisms, including those used as seafood, are also a source of organic compounds of potential medical importance. Within the framework of an antimicrobial activity study of marine macro-organisms from the Persian Gulf, bioactive compounds of the sea cucumber *Holothuria leucospilota* were extracted from whole sea cucumber bodies using chloroform or methanol. The extracts were evaluated for their antibacterial effects against methicillin-resistant *Staphylococcus aureus* (MRSA) and enterotoxin producing *Staphylococcus aureus strains* (SEASA, SEBSA). Antimicrobial activities were determined using three methods: disk diffusion tests, minimum bactericidal concentration (MBC), and minimum inhibitory concentration (MIC). The results demonstrate that methanol and chloroform extracts have an inhibitory effect on the growth of all strains tested at MIC concentrations up to 100 mg/ml. Also, the chloroform extract demonstrated

bactericidal activity against SEBSA in concentrations of about 100 mg/ml. The extract below 100 mg/ml concentrations also shows bactericidal effects against MRSA and SEBSA. The highest antibacterial activity was found in the methanol extract. Therefore, sea cucumber extract is an interesting candidate for the identification of new antimicrobials, however, comprehensive investigations are needed to separate and identify the active compounds from *Holothuria leucospilota* from the Persian Gulf.

Keywords: Sea cucumber, Antimicrobial activity, *Staphylococcus aureus*

1.Introduction

The biodiversity of the marine ecosystem surpasses that of the terrestrial ecosystem (1) and therefore the number of exploratory studies searching for compounds of pharmaceutical interest is steadily growing. Sea cucumbers, echinoderms from the Holothuroidea class, are slow-moving, primarily nocturnal and benthic invertebrates(2). They have been an essential source of food and used in traditional medicine, especially in some parts of Asia(3) and contain a rich profile of bioactive molecules of therapeutic potential for anti-cancer (4,5) anti-tumor and anti-angiogenic (6,7) antimicrobial, and antioxidant compounds(8) .Interesting bioactive compounds include lectins, sterols, peptides, cerebrosides, glycoprotein, glycosaminoglycan, glycosphingolipids, sulfated polysaccharides, and essential fatty acids (4,9).

The Persian Gulf (coastal waters of Iran) is a unique environment rich in biodiversity (10). *Holothuria leucospilota*, ordinarily known as the black tarzan or black sea cucumber, is the most frequently harvested sea cucumber in the Persian Gulf, starting in 2004 at Qeshm Island (11). *H. leucospilota* primarily generates bioactive secondary metabolites, making it attractive for drug discovery (3). Because of the increasing need for food and drug candidates, sea cucumbers have been increasingly studied (12,13). However, out of nearly 1400 articles recorded globally concerning *Holothurian* species (10,11), only about 20 investigated *Holothurian* species found in the Persian Gulf. Thus, there is still the opportunity for scrutinizing the biological and antimicrobial activity of sea cucumber populations in this region (14). Previous studies demonstrated mild antibacterial efficacy of *Holothurian* compounds in very high concentrations (15,16), For example, extracts from various organs or tissues of the sea cucumber *Holothuria* displayed limited bacteriostatic activity eluded with methanol, chloroform, or n-hexane eludes when tested against human pathogens(11). Compared with

conventional antimicrobials, purified Holothurian peptides employed as antibacterial agents displayed relatively weak effectiveness against *S,aureus* (7). Interestingly, protein hydrolysates of sea cucumbers are considered as a healthy and reliable substitute for artificial food preservatives (17,18), suggesting antimicrobial activity.

Due to the production of distinctive naturally occurring bioactive compounds, research into aquaculture drug sources such as the sea cucumber *H. leucospilota* is necessary. The medicinal and antibacterial effects of sea cucumbers are well-known. We investigated the hypothesis that the antibacterial activities of methanol compared to chloroform extracts from the whole body of *H. leucospilota* against methicillin-resistant *Staphylococcus aureus* (MRSA) and enterotoxin *producing Staphylococcus aureus strains* (SEAA, SEBSA). The activity is determined by disc diffusion tests, MIC, and MBC using micro dilution.

2. Materials and Methods

2.1. Sample collection

A collection of healthy and comparable sea cucumber *Holothuria leucospilota* were carefully collected from the Quesm Island station located in the Persian Gulf.. Samples were maintained at -20°C until used.

2.2. Extraction and isolation of the samples

Bioactive compounds were extracted based on their polarity using water and organic solvents. After defrosting *H. leucospilota*, they were cleaned with fresh water and cut from their anus to their mouth (2). The whole bodies were opened, split into several pieces, and then kept at +45°C for two days to completely dry. This process is called the scale method (2). Next, the dehydrated samples were crushed using a grinder (WordStar) and were powdered thoroughly. The powder was extracted using a soxhlet extractor with either chloroform or methanol as solvents (Merck, Darmstadt, Germany) for 6 hours. Then, following (19), the solvents were evaporated under vacuum conditions. Finally, the extracts were freeze-dried to completely remove solvents and to create a solid form with increased purity.

2.3. Antibacterial assay

All staphylococcal strains (MRSA, SEASA, and SEBSA) were tested with suspensions equivalent to 0.5 McFarlands (20,21) ATCC 43300 (MRSA positive), ATCC 25923 (*sea*

positive), and ATCC 14448 (*seb* positive) were used in the experiments (19,21). The samples were cultured in Mueller Hinton Broth (MHB) (Merck, Darmstadt, Germany) and incubated at 37 °C for 6 h. The methanol and chloroform extracts of the whole body were assayed using disk diffusion(20) ,The disk diffusion methodology is commonly used for antimicrobial susceptibility of *S. aureus* (20,22). Keeping the overall concentration at 100 mg/ml, we added 40 μg/mL of methanol extract or 40 μg/mL of chloroform on a blank disk. Positive controls were vancomycin and clindamycin which were incubated at 37 °C for 24 h to measure the zones of inhibition (the standard methods CLSI document 2010; M100-S20).

The antimicrobial activity of *H. leucospilota* extract was also evaluated by the minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC). In our study, the extract optical density (OD) was read at times (measured in hours, h) 0 h, 4th h, 8th h, 12th h, and 24th h using an ELISA reader (BioTek, USA) at 650 nm. In case that the OD was decreased, it indicates that the extract had an inhibitory effect on bacterial growth Samples in the multi-well plates that did not increase in OD after 24 h were examined for MBC (20). As controls, the same strains were used without methanol or chloroform extract. All experiments were run in triplicate.

2.4. Statistical analysis

SPSS 19.0 (IBM, SPSS) was used for the analysis of variance of the raw data. Using Duncan's multiple range tests in ANOVA, we report all data as mean±SD. We also perform the least significant difference (LSD) (N = 12) tests to compare results among multiple groups ($p<0.05$) (Total df:39, time –concentration df:1) Table 1 and Table 2.

3. Results

3.1. Disc diffusion

The highest antibacterial activity in the disk diffusion assay was determined in the methanol extract against MRSA (Table3). Compared to the zones of inhibition of the positive controls vancomycin and clindamycin, the methanol extract displayed a higher growth inhibition against MRSA than the chloroform extract. The chloroform extract showed still inhibitory effectiveness against both MRSA and SEBSA. By comparing the performance of extract and antibiotics, we find that the extracts are effective against *S. aureus*, but less so than the pure antibiotics (Table 3).

3.2. Antibacterial assays

The results of the antibacterial assays using the methanol and chloroform extracts from the whole body of *H. leucospilota* against staphylococcal MRSA, SEASA, and SEBSA are shown in Table 4. The results confirm bacteriostatic rather than bactericidal effects and show significant differences (max $P<0.05$; min $F>9.3$) between the means of antibacterial activity of extract of various concentrations in both MIC and MBC in the studied sea cucumber *H. leucospilota*. In the antibacterial assays, the methanol extract showed significant activity against SEASA and MRSA with MICs around 100mg/ml (Fig. 1). The highest activity was exhibited with MICs ranging from 100 to 25mg/ml of methanol extract against SEBSA (Fig.1). In the methanol extract, bactericidal activity has been found against SEBSA and MRSA with MBCs that range around 100 μg/ml (Table 4). Chloroform extracts from the whole body showed antibacterial activity against MRSA, SEASA, and SEBSA with MICs of about 50 mg/ml (Fig.2 panels a, b, and c). Also, chloroform extract demonstrates bactericidal activity against SEBSA at concentrations of about 100 mg/ml (Table 2).

4. Discussion

In this study, *H. leucospilota* methanol extract showed inhibitory activity towards the tested *S. aureus* strains MRSA and SEASA around 100 mg/ml. Additionally, the methanol extract has been shown to be efficient against SEBSA strains with MICs ranging from 25 to 100 mg/ml. The chloroform extract is efficient against MRSA, SEASA, and SEBSA strains in MICs ranging from 50 mg/ml to 100 mg/ml and effective against SEBSA in MBCs below100 mg/ml. We detected the highest antibacterial activity in methanol extract with a zone of inhibition of 14 mm against MRSA. This could be due to the better suitability of methanol as a solvent for the bioactive products generated across different organs in *H. leucospilota*.

Microorganisms utilize different means to limit the concentration of antibacterial agents inside cells: decreasing diffusion, increasing efflux or neutralization of the antibacterial agents that may reversibly or non-reversibly render the drug inactive). In most of the studied species of sea cucumbers (14,22,23,24,25) either the body walls or the whole bodies were examined. In addition, limited antimicrobial activity of holothurian compounds has been shown under very high concentrations (26). It should be emphasized that other species or populations of sea cucumber may have different levels of activity than in our study. For example, Yasoda *et al* (2006) studied methanol-acetone extract obtained from sea cucumber body wall of

Parastichopus parvimensis, in Santa Catalina Island, California, on *Escherichia coli* by disc diffusion. In their study, two antibiotics were used as a positive control, ampicillin and tetracycline. The antibacterial effect of the extract *on Escherichia coli* were confirmed. The zone of inhibition of the extract though was much smaller than those of the antibiotics. In addition, in tests with methanol and chloroform extracts sampled from various tissues and organs of *H. leucospilota*, moderate bacteriostatic activity against a few selected species of Gram-negative and Gram-positive bacteria was reported (2). Nevertheless, bacteriostatic effects against *Shigella dysenteriae*, *Proteus vulgaris*, *Bacillus cereus*, *S. epidermidis* and *Candida albicans* were not noted in such high concentrations (26). In previous studies, it was suggested that the active compound was a lysozyme (18). In our study, the whole-body extracts of *H. leucospilota* exhibited antimicrobial activities against MRSA, SEASA, and SEBSA, with potentially varied explanations. Therefore, comprehensive chemical analyses are needed to isolate and purify the active compounds, identify their chemical nature, and evaluate their possible suitability for new drug leads (2,26) analyzed methanol, ethyl acetate, and water-methanol extracts taken from the body wall of sea cucumbers from the Persian Gulf on *S. aureus, Escherichia coli,* and *Pseudomonas aeruginosa*. However, they did not detect any inhibitory effect of the extracts on bacterial growth. (2) studied the antimicrobial activity of hexane, chloroform, and methanol extracts from the body wall, gonad, and intestine of *H. leucospilota* against *S. aureus, Bacillus subtilis,* and *P. aeruginosa*. *P. aeruginosa* is the most sensitive bacterium to the extracts, with no antibacterial effect being seen under any concentration on *B. subtilis and S. aureus.* It is currently unclear what exactly explains the differences in our results and the existing literature. Other possible reasons include different extraction techniques, different solvents usage, and the ecological conditions of the studied specimens.

A critical note is the number of samples and extracts in this study. While we have three strains and two pooled extracts (one with chloroforms and one with methanol), these strains have great importance and are used in related studies. In addition, although the amount of pooled samples prohibits a thorough statistical analysis from reflecting the variation in sea cucumbers, it should be enough to provide a robust general insight. Nevertheless, for comprehensive statistical analysis, we recommend a higher number of samples and extracts for future studies.

5. Conclusion

Sea cucumbers are well-known for their pharmaceutical value due to their production of distinctive natural bioactive compounds. In this study, whole-body extracts of *H .leucospilota* collected from the Persian Gulf showed antibacterial effects against *S. aureus strains* MRSA, SEASA**,** and SEBSA. Future studies are required to focus on the chemical composition and mechanisms of action. Moreover, investigations on possible aquaculture of potential sources of drugs such as sea cucumber *H. leucospilota* are necessary.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Acknowledgment

N.A. is extremely grateful to Prof. Dr. Jens Rolff for his insightful comments on and editing the paper at various occasions. We are thankful to Dr. Charlotte Rafaluk-Mohr for her help with language proof reading. The usual disclaimer applies. A preprint has previously been published(28).

Author Contributions

N.A. designed the study and conducted the experimental work, M.M.K. led the statistical analysis with supports from N.A., N.A. wrote the main manuscript text and both authors reviewed the manuscript and participated in the revision.

Competing interests

The authors declare that they have no conflicts of interest.

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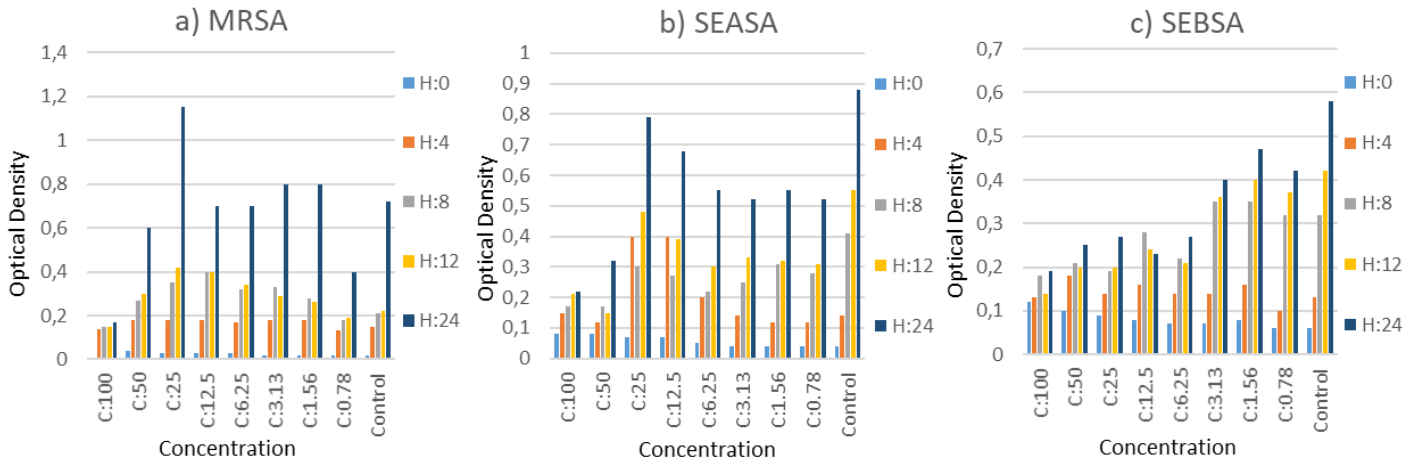
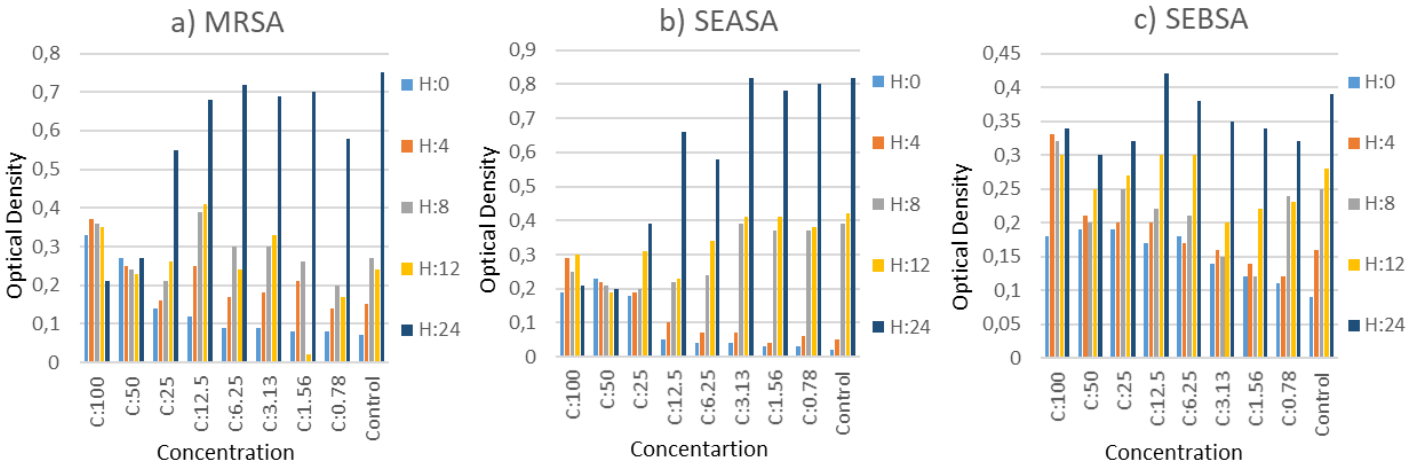


Concentration (mg/ml). Hours (H).

Figure 1. The antimicrobial effect of methanol extract of (N = 12) sea cucumbers *H. leucospilota* at different times (0,4,8,12,24 hrs) and concentrations. Optical density of extract was read by the ELISA reader at 650 nm. Concentrations of the methanol extract showed minimum inhibitory towards a) the tested *S. aureus* strain MRSA with statistical results (max $p<0.05$; min $F>13.3$), b) *S. aureus* strain SEASA with statistical results (max $p<0.05$; min $F>14.1$), and c) *S. aureus* strain SEBSA with statistical results (max $p<0.05$; min $F>17.0$),)(df total :39 ,df time –concentration:1) all with MICs of about 25 mg/ml. values. The strains are ATCC 43300 (MRSA positive), ATCC 25923 (*sea* positive), and ATCC 14448 (*seb* positive), which were applied in the experiment without methanol or chloroform extract. 12 sea cucumber extracts were combined, and all experiments were run in triplicate.

Strain	Time (hours)	Concentration (mg/ml)	Statistical Results (p-value, F-value)
MRSA	0 h	25	$p<0.05$; $F>13.3$
SEASA	4 h	25	$p<0.05$; $F>14.1$
SEBSA	8 h	25	$p<0.05$; $F>17.0$

Table 1. Illustrates the antimicrobial impact of the methanol extract from sea cucumbers *(Holothuria leucospilota*) at diverse concentrations and time points. The optical density of the extract was measured at 650 nm using an ELISA reader. The concentrations of the methanol extract demonstrated significant minimum inhibitory effects against *S. aureus* strains MRSA, SEASA, and SEBSA, each with distinct statistical outcomes (max $p<0.05$; min $F>13.3$, max $p<0.05$; min $F>14.1$, max $p<0.05$; min $F>17.0$, respectively))(df total :39 ,df time –concentration:1), all exhibiting minimum inhibitory concentrations (MICs) of approximately 25 mg/ml.



Concentration (mg/ml). Hours (H).

Figure 2. The antimicrobial effect of chloroform extract of (N = 12) sea cucumbers *H. leucospilota* at different times (0,4,8,12,24 hrs) and concentrations. Optical density of extract was read by the ELISA reader at 650 nm. Concentrations of the chloroform extract showed minimum inhibitory towards a) the tested *S. aureus* strain MRSA with statistical results (max $p<0.24$; min $F>1.4$) and ($R2=80.6\%$), b) *S. aureus* strain SEASA with statistical results (max $p<0.05$; min $F>13.9$), and c) *S. aureus* strain SEBSA with statistical results (max $p<0.05$; min $F>9.3$)(df total :39 ,df time –concentration:1) all with MICs of about 50 mg/ml. values. The strains are ATCC 43300 (MRSA positive), ATCC 25923 (*sea* positive), and ATCC 14448 (*seb* positive), which were applied in the experiment without methanol or chloroform extract. 12 sea cucumber extracts were combined, and all experiments were run in triplicate.

Strain	Time (hours)	Concentration (mg/ml)	Statistical Results (p-value, F-value)
MRSA	0	50	$p<0.24$; $F>1.4$
SEASA	4	50	$p<0.05$; $F>13.9$
SEBSA	8	50	$p<0.05$; $F>9.3$

Table 2. investigated the antimicrobial effects of the chloroform extract obtained from sea cucumbers (*Holothuria leucospilota*) at various concentrations (50 mg/ml) and time points (0, 4, 8, 12, 24 hours) against *Staphylococcus aureus* strains MRSA, SEASA, and SEBSA. (df total :39 ,df time –concentration:1) Optical density readings at 650 nm were recorded using an ELISA reader. The chloroform extract demonstrated minimum inhibitory concentrations (MICs) of approximately 50 mg/ml for all tested strains. Statistical analysis revealed significant inhibitory effects, with strain-specific results including maximum p-values below 0.24 and minimum F-values exceeding 1.4.

Table3. Disk diffusion result

Staphylococcus aureus	**Chloroform extract 40 µg/mL**	**Methanol extract 40 µg/mL**	**Vancomycin 30 µg/ml**	**Clindamycin 25 µg/ml**
MRSA	12 (mm)	14 (mm)	18 (mm)	16 (mm)
SEASA	9 (mm)	10 (mm)	19 (mm)	18 (mm)
SEBSA	12 (mm)	11 (mm)	18 (mm)	18 (mm)

Table 3. Inhibition zones for *S. aureus* strains MRSA, SEASA, and SEBSA under the effect of different extract of *H. leucospilota* with concentrations of 40 µg/mL and vancomycin, clindamycin by disk diffusion test. ≥17mm is sensitive zone for Vancomycin30 µg/ml and ≥ 14mm is sensitive zone for Clindamycin 25 µg/ml.

Table4. Antimicrobial activity of whole body of *H. leucospilota* methanol and chloroform extract

Staphylococcus aureus	***H. leucospilota* Extract**	**MIC**	**MBC**
MRSA	Methanol 40 µg/mL	>25 mg/ml	100 mg/ml
SEASA		>25 mg/ml	-
SEBSA		>25mg/ml	100 mg/ml
MRSA	Chloroform 40 µg/mL	>50 mg/ml	-
SEASA		>50 mg/ml	-
SEBSA		>50 mg/ml	100 mg/ml

Table4. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) from the whole body of *H. leucospilota* methanol and chloroform extract against *S. aureus* strains MRSA, SEASA, and SEBSA.

CHAPTER III: Identification of *Vibrio kanaloae* in the oyster *Crassostrea gigas* by fluorescent in situ hybridization

Abstract

An ideal model for studying *Vibrio* species and and their interaction with hosts are disease dynamics in the oyster *Crassostrea gigas* in the North Sea, exposing invasive sources to pathogens, especially (*Vibrio kanaloae*). This study used molecular fluorescent in situ hybridization (FISH) techniques to rapidly identify the diversity of bacteria in the oyster *Crassostrea gigas* from the island of Sylt and 16SrRNA gene sequence validated all strains' identification. Oysters were collected and exposed to *Vibrio kanaloae* strains , one allopatric T02 and one sympatric S12 anddifferent tissues were examined utilizing culture-independent methodologies. The digestive glands, gill and Muscle in *vibrio kanaloae* were identified as metabolically active by FISH technique. One of the advantages of the FISH technique is that it is accurate . Both *Vibrio* strains S12 and T02 were detected in the gill tissue, but with a very low level of colonization. The numbers of *Vibrio* strains observed in the digestive glands were found to be comparable, with just a small number of bacteria seen. It is notable that the detection of *V. kanaloae* strains S12 and T02 in the muscular tissue of oysters was seen seldom or with limited prevalence, suggesting a possible discrepancy in their temporal distribution throughout the oyster organism. This technique allows rapid assessment of *Vibrio* in oysters. Recognizing the fundamental difficulties linked to probe specificity, including the possibility of non-specific binding or cross-reactivity, we implemented a rigorous approach in our methodology. Experiments that were controlled, particularly those that incorporated negative controls, were scrupulously executed in order to identify and reduce occurrences of false positives. It is crucial to emphasize that the systematic alignment of the consistent detection of *Vibrio* with the parameters of our experimental infection paradigm strengthens the reliability and applicability of our results.The research underscores the efficacy of

CHAPTER III: Identification of *Vibrio kanaloae* in the oyster *Crassostrea gigas* by fluorescent in situ hybridization

FISH in evaluating the dynamics of *Vibrio* in oysters, shedding light on the interplay between *Vibrio* and the host-pathogen relationship, and establishing a foundation for subsequent investigations.

Keywords

Vibrio kanaloae, oyster *Crassostrea Gigas*, Fluorescent in situ hybridization

Introduction

Bacterial diseases are limiting factors for sustainable aquaculture development caused by the associated opportunistic microbiota. Members of the microbiota can become pathogenic to breeding organisms when stressed due to insufficient feeding or management methods, high stocking densities, and bad environmental conditions (1). Infection together with ecological and physiological imbalances can result in massive mortality a (2,3,4).In marine environments, *Vibrio* bacteria are abundant, and some pathogenic species can cause disease in invertebrates and vertebrates, including humans (5). In some cases, it has been reported that in the aquaculture industry, *Vibrio* infections have caused severe production losses with significant economic effects, including the following: mass mortality in shrimp (6,7,8,9) fish (10) and shellfish (11,12,13,14). One important organism in aquaculture, is the oyster *Crassostrea gigas*. Over six million tons per year are harvested (15). The summer mortality syndrome (SMS) and other Pacific oyster mass mortality began in the 1960s and have spread to many countries around the world, such as Japan (16), the United States (17,18), France (19), and the southern part of the North Sea (20). *Vibrio* spp. are found in most marine ecosystems and are adaptable to various lifestyles, such as free-living, opportunistic, pathogenic forms, or mutualistic (21, 22,23). *Vibrio* isolation from moribund and healthy *C. gigas* has been performed over 30 years (24,25,26,27,28). The species predominant involved in mortality of oyster were: *V. tubiashii, V. anguillarum*, *V. splendidus*, and *V. aestuarianus* (12,13,23-27). In recent years, bacterial communities have been detected, identified, and quantified using 16S rRNA/rDNA in various specimens by PCR (polymerase chain reaction)

and using FISH (fluorescent in situ hybridization) (1,29,30). Most studies on oysters have been related to culturable bacteria, while marine bacteria often lack cultivability (31,32). Few molecular methods reports describe the diversity of bacteria in seemingly healthy oysters (33,24,35). However, significant progress has been made in identifying the pathogenic species of *Vibrio*. The dataset for this study was gathered during the summer, employing meticulously controlled infection tests on oysters to assess the pathogenicity of locally prevalent strains at elevated temperatures (3,28). However, a substantive gap persists in comprehending the bacterial composition and diversity within these bivalves. Insight into the pathological and physiological dimensions, as well as the ecological interactions between oysters and indigenous bacteria, assumes paramount importance in averting consequential bacterial diseases in aquaculture (29).This investigation endeavors to bridge this knowledge lacuna by delineating the temporal dynamics of *Vibrio* during oyster infections. Notably, the nuanced ecological interactions between oysters and the indigenous bacterial community remain inadequately characterized. Hence, an in-depth exploration of bacterial composition and diversity in these bivalves becomes imperative. To this end, the study seeks to explore the utility of Fluorescent In Situ Hybridization (FISH) as a method for the swift, facile, and accurate detection of *Vibrio kanaloae* in *Crassostrea gigas*. This methodological pursuit is underpinned by the culmination of data amassed during the summer, particularly stemming from experimental inquiries into the pathogenicity of local strains under conditions of heightened temperature through controlled infection tests on oysters (3,28).The outcomes of this investigation hold promise in advancing our comprehension of the intricate interplay between oysters and *Vibrio* bacteria, thereby contributing substantively to the prevention of bacterial diseases in the realm of aquaculture.

Material and methods

Infection experiments

Healthy oysters (showing no disease signs) were obtained in August 2020 (three weeks before testing) from a site in the North Sea island Sylt (5582.330 N, 8826.570 E). The oysters were adapted to the experimental temperature of 20 °C at a fixed temperature (room temperature change

during adaptation per day was less than 0.58 °C) Wendling CC, Wegner KM.2013 and 2015 *et al* (28,37). The oysters were maintained in a flow system. The oysters were cleaned of epibionts a week before the test, and the small shell side (related to the muscle of the adductor stick) was cut by a small hand drill. In this study, two *V. kanaloae* closely related isolates were selected from any site (T02 from Texel and S12 from Sylt, as described by Wendling CC, Wegner KM. *et al* ,2013 (37). *V. kanaloae* bacteria are involved in *Vibrio* isolates sampled from the haemolymph of the oyster. For geographical reference, the strains are known as *Vibrio* North and *Vibrio* South. In total, 12 oysters were used in this study. In a fixed climate chamber, oyster water temperature was kept at 20 °C, and the oysters were placed separately in 1 liter aerated glass jars. Water was changed once every other day. For every test group, four oysters with *Vibrio* north or south were used. For each control group, four oysters with nutrient solution 1.5% were applied. The protocols of infection presented in Wendling and Wegner *et al* (2013) (38) were used for treatments. Briefly, using a syringe, $10^{\wedge 8}$ bacterial culture cells or an equal nutrient solution volume of 1.5% NaCl were pre-injected into the muscle of the adductor using the predrilled hole. In 3rd day Tissue samples were obtaining from the oysters.

FISH (fluorescence in situ hybridization) and specimen processing

Tissue samples were collected from the muscle, gill, and digestive gland of oysters at the same site to ensure the bacterial population remained unchanged and cellular activity was not lost(29). The tissue samples experienced standard FISH diagnostics at the University of Charite using a solution containing 3.7% formaldehyde in phosphate buffer saline (pH 7.4) and 50% ethanol at 4 °C. Following this, the samples were placed in cold polymerizing resin (39). For hybridization, 20 μL of a hybridization solution was heated and then mixed with five pmol of the respective oligonucleotide probe and 4',6-diamidino-2-phenylindole (DAPI) particular nucleic acid staining and attentively used to tissue sections. The probes were tagged with 5'-ends with FITC (fluorescein 5-isothiocyanate) (EUB338) or endo carbocyanin Cy3 (AURI) and Cy5 (NONEUB338) (Biomers, Ulm, Germany). They were then incubated for 2 hours in a chamber at 50 ^{0} C. After this step, the slides were washed with water, immediately dried in the air, and installed with an installation medium to protect the fluorescence signal from fading (Vectashield, Vector Laboratories, California, USA). An epifluorescence microscope (Axioplan2; Carl Zeiss, Jena, Germany) with a

set of namowband filters (AHFAnalysentechnik, Tu bingen, Germany) was used for the microscopy.

Oligonucleotide probes

In most domain bacterial microorganisms applied to bacterial colonization screen, the probe EUB338 (5'GCTGCCTCCCGTAGGAGT-3) (39,40) complements a portion of the 16S rRNA gene conserved. The EUB338 antisense probe was used to remove the non-specific probe connection from the NONEUB338 probe. A panel of FISH probes was also used to detect common species in IE (41).

Bacterial strains

These *Vibrio kanaloae* strains were either isolated in the same location as the oysters S12 (sympatric) or in a different location T02 (allopatric). Allopatric and sympatric strains were used for positive control ,Wendling CC, Wegner KM.2013 and 2015 *et al*. As the closest phylogenetic neighbor with three mismatches at the probe junction, *Vibrio* spp. was applied to establish a negative control to improve particular Fish conditions for endo carbocyanin Cy3 (AURI). The strains of bacteria were fixed for negative and positive controls (43) and contained in every FISH test to control the sensitivity and exclusivity of the probe.

Result

We found that *Vibrio* sympatric strains S12 were detected in digestive glands, gills, and muscle tissues three days post-infection (Figure 1). The study found that allopatric strain T02 had a few colanizations in gill tissue and similar amounts in digestive glands (Figure 2). However, the detection of these strains in muscle tissue was rare or minimal. (Figure 1 and 2). The findings highlight the importance of FISH in evaluating Vibrio populations in oysters.

CHAPTER III: Identification of *Vibrio kanaloae* in the oyster *Crassostrea gigas* by fluorescent in situ hybridization

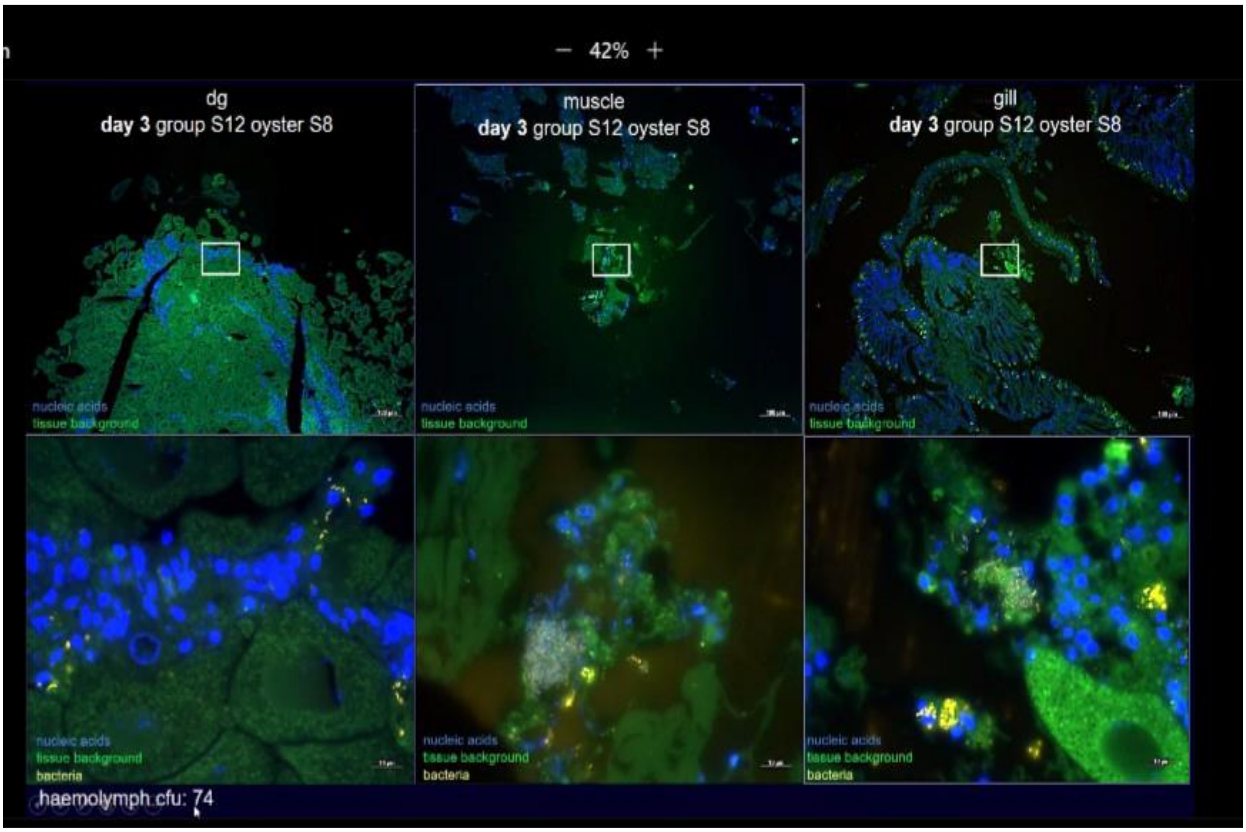


Figure 1: Fluorescence in situ hybridization (FISH)microscopic analysis of *Vibrio kanaloae* sympatric Strains S12 in Oyster Tissues on Day 3 Post-Infection. Employing Fluorescence in situ Hybridization (FISH), this figure captures the spatial distribution of *Vibrio kanaloae* sympatric strains S12 within oyster tissues three days' post-infection. (A) The fluorescence signals, generated by the pan-bacterial FISH probe EUB338 (blue), vividly illustrate the infiltration of *Vibrio* sympatric strains S12 in the gill, digestive gland (df), and muscle tissues of the oyster. The background fluorescence in green provides context to the tissue. Zooming in on the inset (B), This visual representation offers an insight into the microbial colonization dynamics during the early stages of infection in oyster tissues.

Figure 2. This figure illustrates the outcomes of the Fluorescence in situ Hybridization (FISH) technique, providing *Vibrio kanaloae* colonization in oyster tissues three days after infection. The results reveal differential patterns in DAPI staining and pan-bacterial FISH probe signals across distinct tissue types.

(A) Gill Tissue: DAPI staining showed a few positive signals, indicating the presence of microbial DNA. The pan-bacterial FISH probe demonstrated positive signals, suggesting active colonization by *Vibrio kanaloae*.(B) Digestive Gland (df) Tissue: Similar to the gill, DAPI staining indicated a few positive signals, and the pan-bacterial FISH probe revealed positive signals, indicating *Vibrio kanaloae* colonization.(C) Muscle Tissue: DAPI staining exhibited rare positive signals, suggesting a limited presence of microbial DNA. In contrast, the pan-bacterial FISH probe showed negative signals, indicating a lack of *Vibrio kanaloae* colonization in the muscle tissue.

Discussion

The objective of this study was to detect the colonization of *Vibrio kanaloae* bacteria colonization in the oyster species *Crassostrea gigas* from Sylt Island. This was achieved by the utilization of fluorescent in situ hybridization (FISH) technique, followed by the validation of the identification using 16SrRNA gene sequencing. The study focused on the examination of the detection and separation of *Vibrio kanaloae* strains allopatric T02 and sympatric S12 in different oyster tissues, specifically the digestive glands, gill, and muscle, three days post-infection. The utilization of this technique facilitated rapid evaluation of *Vibrio* species within the oyster . The investigation showed identifiable colonization patterns by strains of *V. kanaloae* inside different oyster tissues. Both *Vibrio* strains S12 and T02 were found in the gill tissue, but with a very low population size. The digestive glands exhibited similar amounts of both *Vibrio* strains, with just a few bacteria detected. Interestingly, the detection of *V. kanaloae* strains S12 and T02 in the muscle tissue of oysters was found to be rare or little seen, indicating a potential disparity in their temporal distribution inside the oyster organism .Aquatic products consumption has increased in recent years, which has raised public health concerns about more sensitive, faster, and more specific pathogen detection (46). However, cultivation methods are used to identify *Vibrio* spp. This is time-consuming and takes four to seven days. Therefore, using molecular techniques as the method of choice for rapid diagnosis of *Vibrio* strains would be beneficial (47-50). PCR as an essential molecular method is commonly used extensively in the detection of bacteria (51,52,53). DNA microarrays have also been reported in the *Vibrio* diagnosis (54,55). The FISH method was used to detect *Vibrio* strains in this study by probing Vib- 16S-1 with 16S rRNA. Species of *Vibri*o that are present in marine environments and seafood can be identified in FISH assays (56). The short hybridization process (only 1 -2 hours) and the quick and easy fixation of bacteria on the slides are other components of this method. The entire diagnostic procedure is usually performed within two days (Almost three days less than conventional biochemical methods). Unlike PCR and other molecular methods, this method provides results without extracting DNA from bacteria. In the case of food, DNA extraction is relatively hard because of the matrix's complexity and the DNA low abundance (56). The FISH method is, however, less sensitive than PCR methods due to the lack of DNA amplification. Further improvements are required to optimize the conditions of culture and formulate a global enrichment medium to recover good strains of *Vibrio* from

specimens range with competing flora levels. According to the information obtained, the Vib-16S-1 probe can detect the dominant pathogenic species of Vibrio. The identification time can be significantly decreased than culture-based methods because of the bacteria direct visualization. The findings of this study emphasize the value of Fluorescence In Situ Hybridization (FISH) as a helpful technique for the prompt and accurate evaluation of *Vibrio* colonization in *Crassostrea gigas*.

Conclusion

The use of FISH techniques in *Crassostrea gigas* adds to the study on the application of molecular methods for detecting *Vibrio* and other infections in oysters and mollusks. The combined use of FISH and 16SrRNA gene sequencing, along with the consideration of tissue-specific metabolic activity and temporal dynamics, contributes to a more nuanced understanding of the complex interactions between *Vibrio* species and their molluscan hosts. This contextualization within the current investigation enhances the significance of our findings and provides a foundation for future research in the field.

Acknowledgment

We extend our sincere gratitude to Dr. Mathias Wegner of the Alfred Wegener Institute (AWI) for his invaluable contribution to the experimental design and execution focused on the dynamics of *Vibrio* species in *Crassostrea gigas* from the North Sea. His expertise in oyster experiments and insightful guidance significantly enriched this study.We also wish to express our appreciation to Prof. Dr. Annette Moter and her group at Charité Medical University for her expertise in employing molecular fluorescent in situ hybridization (FISH) techniques. The meticulous application of FISH allowed for the rapid identification of *Vibrio kanaloae* strains within different tissues of *Crassostrea gigas*. Prof. Dr. Moter's dedication and expertise have been instrumental in advancing our understanding of the host-pathogen relationship and disease dynamics.Their collaborative efforts and expertise have played a pivotal role in the successful execution of this research. We are grateful for their mentorship, guidance, and commitment to advancing scientific knowledge in the field.

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The co-evolutionary context of immune priming and cross-protection in the oyster *Magalana (Crassostrea) gigas*

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Infections with pathogenic *Vibrio* strains are associated with high summer mortalities of Pacific oysters *Magalana (Crassostrea) gigas*, affecting production worldwide. This raises the question of how *M. gigas* cultures can be protected against deadly *Vibro* infection. There is increasing experimental evidence of immune priming in invertebrates, where previous exposure to a low pathogen load boosts the immune response upon secondary exposure. Priming responses, however, appear to vary in their specificity across host and parasite taxa. To test priming specificity in the *Vibrio – M. gigas* system, we used two closely related *Vibrio splendidus* strains with differing degrees of virulence towards *M. gigas*. These *V. splendidus* strains were either isolated in the same location as the oysters (sympatric, opening up the potential for co-evolution) or in a different location (allopatric). While addition of haemolypmph plasma in general promoted growth of both strains, priming by an exposure to a sublethal dose of bacterial cells lead to inhibitory effects against a subsequent challenge with a potentially lethal dose *in vitro*. Inhibitory effects of oyster haemolymph on *Vibrio* growth was detected *in vitro* when oysters had been primed with sympatrically isolated *Vibrio*. Inhibitory effects were seen both when challenged with the sympatric as well as against allopatric *V. splendidus*, suggesting some degree of cross protection. Challenges with sympatric *V. splendidus* led to immune priming in oysters most strongly when they were

rechallenged with the sympatric strain, suggesting that matching local strains provides the highest level of protection and could prevent vibriosis.

Keywords: *Vibrio splendidus*, oyster *Crassostrea gigas***,** Immune priming, cross-protection

Introduction

According to the Food and Agriculture Organization of the United Nations (FAO), in 2019, global aquaculture production, which includes fish, oysters, other molluscs, crustaceans and other aquatic species for human consumption, exceeded 177,8 million metric tons, an increase from 147.2 million metric tons in 2010 (Food and Agriculture Organization (FAO) and WHO Report 2010, FAO 2020). Aquaculture is a significant contributor to the global food fishery industry (FAO and WHO 2010 ,FAO, 2020), however, infectious diseases increasingly cause problems for shellfish and especially oyster aquaculture (Lafferty, K.D., Harvell ,2015).

In particular, bacteria of the genus *Vibrio* are recognized as important infectious agents impeding aquaculture development (Paillard, C *et all* 2004, Le Roux, F, *et all* 2016,Ina-Salwany, *et all* 2019)*Vibrios* are gram-negative bacteria that are widespread in marine and estuarine ecosystems and can be found free-living in the water column, as part of biofilms, or in association with a host (Thompson, F.L,*et all* 2004 ,Austin, B., and D.A. Austin. 2007). *Vibrio* species are causal agents of epizootics, zoonoses, and epidemics, with several *Vibrio* species causing disease in humans (LeRoux, F. *et all* 2015). *Vibrio* strains from the S*plendidus* clade in particular (e.g. *Vibrio tasmaniensis* and *Vibrio crassostreae*) have been associated with mortality in oysters (Lopez-Joven C, *et all* 2018 , Rubio T, *et all* 2019). Furthermore, *Vibrio* contributes to Pacific oyster mortality syndrome which effects the food oyster species *Magalana gigas and* occurs seasonally when seawater temperature reaches 16-24 C (Petton *et al.*, 2021).

Several oyster species are grown in aquaculture globally and play essential roles in coastal ecosystem functioning. *M. gigas*, in particular, has become a model for studying the dynamics of *Vibrio* disease in wild animals (Guo, X.M *et al.*, 2015)1. Oysters, like other invertebrates, have

evolved an integrated, highly complex innate immune system to identify and eliminate various invaders through a variety of orchestrated immune responses (He, X., Zhang *et al.*, 2011b). These reactions consist of immune recognition, the synthesis of antimicrobial peptides, signal transduction and encapsulation and phagocytosis of the circulating hemocytes ((He, X., Zhang *et al.*, 2011b). The hematopoietic tissue, hematopoiesis and circulating hemocytes have been mostly characterized, and the detailed annotation of the Pacific oyster genome has revealed massive expansion and functional divergence of innate immune genes in this species (Kurtz, J., Franz, K., 2003). The immune response of invertebrates may also exhibit adaptive-like characteristics, referred to as immune priming, as demonstrated phenotypically (Lemaitre, B,q, *et al* 1997 , Little, T.J. *et al*, 2003 , Pham, L.N *et al* 2007 ,Roth, O., *et al* 2009 , Yue, F., *et al*., 2013, Yue, F., *et al*., 2013,Song, X., *et al* 2016b) and supported by molecular data (Lafont M,, *et al*., 2017 , Martins NE. 2020). Although priming has been demonstrated against viral pathogens in *M. gigas* (Lafont M,., *et al*., 2017 Martins NE. 2020), explicit evidence of priming against bacterial pathogens is more restricted.

There is some evidence of immune priming against bacterial pathogens in *M. gigas*. Fallet et al (2022) tested the impact of environmental microbial exposure on the immune response of oysters, with a specific focus on protection against Pacific oyster mortality syndrome (POMS). They found evidence that prior exposure resulted in higher survival of oysters, not only in the exposed generation but also for subsequent ones. Furthermore, they identified alterations in epigenetic marker resulting in changes in immune gene expression as the causative mechanism. More specifically, there is evidence of changes in the immune system of *M. gigas* following exposure to heat killed *V. splendidus* (Zhang *et al.*, 2014). Total hemocyte counts (THC), number of regenerated hemocytes, and expression levels of hematopoiesis-related genes (e.g. CgRunx1 and CgBMP7) are all significantly increased in oysters following a secondary challenge with *V. splendidus*, suggesting a function for hematopoiesis in immune priming (Zhang *et al.*, 2014). Transfer of serum may enhance immune responses by modulating THC, apoptosis, proliferation, and phagocytosis. After a second challenge with live *V. splendidus*, the phagocytic activity of haemocytes, involving phagocytic rate and phagocytic index, is specifically enhanced in oysters (Zhang *et al.*, 2014). In addition, the expressions of six putative genes involved in the phagocytosis process (CgIntegrin, CgPI3K, CgRho J, CgMAPKK, CgRab 32, and CgNADPH oxidase), are significantly up-regulated

following secondary challenge (Zhang, R *et al*., 2015b). The findings suggest that phagocytosis may play an important role in immune priming. This suggests that if there are benefits in terms of oyster fitness of priming with *Vibrio,* that both humoral and cellular defenses could be involved.

Immune priming can be specific to the bacterial species used for infection (Dhinaut, Chogne and Moret, 2018; Sheehan, Farrell and Kavanagh, 2020) or even the strain, as demonstrated in the snail *B. glabrata* (Portela *et al.*, 2013). Specificity is thought to be linked to the selection pressure and change of re-encountering a specific pathogen (Dhinaut, Chogne and Moret, 2018; Sheehan, Farrell and Kavanagh, 2020). In the *V. splendidus* – oyster system there is evidence of local adaptation of oysters and *Vibrio* strains originating from the same location as the oysters (Wendling, C.C., and Wegner, K.M. 2015, Wegner, K.M *et al.*, 2019).These studies showed that two closely related strains of *Vibrio splendidus* from opposing ends of the Wadden Sea, about 500 km apart, one sympatric isolated from the Northern Wadden Sea on Sylt (Germany) (O7w_July from Sylt) and allopatric one isolated from the southwestern Waddensea on Texel (Netherlands, Tx5.1), had opposing impacts on the immune system activation and survival of oyster isolated at the same location (sympatric) or the other location (allopatric) resembling host-parasite co-evolution by local adaptation (Wendling & Wegner, 2015) or priming by prior contact of oysters to these or closely related strain with similar effectors.

Against the backdrop of potential co-evolutionary adaptations by priming specificity, we now test the impact of a low dose (priming treatment) on *in vivo* pathogen load in Pacific oysters and the inhibitory effect of their haemolymph on *V. spendidus* growth *in vitro* after primary infection. We then went on to secondarily challenge the oysters with a potentially lethal dose of bacteria. For the secondary challenge we either used the same strain as in the primary challenge or the other strain to evaluate local signatures of specificity in immune priming in relation to cross-protection. Comparing the inhibitory effects of oyster hemolymph between sympatric and allopatric strains in a full factorial design allowed us to test whether low dose primary exposures can function as general immunostimulants that enhance the ability of oysters to resist infectious diseases, or whether specificity of the response towards local co-evolved strains needs to be taken into account.

Material and methods

Primary exposure: *in vivo* experiments In August 2021, oysters that were showing no signs of disease were collected three weeks prior to the experiment from a mixed oyster-mussel reef (Oddewatt) on the island of Sylt (5582.330 N, 8826.570 E). Oysters were habituated to the experimental temperatures of 20 C (+/- 0.58 C) in constant temperature rooms. Oysters were kept in a flow-through system supplied with fresh filtered sea water. A week prior to the experiment, oysters were cleaned of epibionts and a small hole was drilled on their dorsolateral sides, closest to their adductor muscles. For infection and priming, we selected two previously described closely related isolates of *Vibrio splendidus* from two different locations. Strain O7w_July came from the same site as the oysters Oddewatt (sympatric) while Tx5.1 was isolated from oysters from Texel at south-western end of the Wadden Sea (allopatric) (Thieltges DW and Wegner, K.M., 2013, Wendling, C.C., and Wegner, K.M. 2015). The strains will be referred to as sympatric *Vibrio* vW from Sylt and allopatric *Vibrio* vT from Texel. Both strains elicited a stronger immune response in their sympatric hosts and consequently induce higher mortalities in their allopatric hosts (Wendling, C.C., and Wegner, K.M. 2015). For infection we used a total of 72 oysters kept individually in aerated glass jars placed in temperature-controlled water baths in a constant climate chamber kept at 20°C. Water was exchanged every two days. For the priming challenge, 24 oysters were infected with sympatric *Vibrio north* O7w_July (group W) and 24 oysters were infected with allopatric *Vibrio south* Tx5.1 (group T) and the remaining oysters with nutrient solution 1.5% NaCl (C group as control) (Figure 1). Treatments followed the infection protocols described in Wendling &Wegner (Thieltges DW and Wegner, K.M., 2013, Wendling, C.C., and Wegner, K.M. 2015). Briefly, we injected 100 µl of 10^4 cells/ml of bacterial overnight culture or an equal volume of nutrient solution 1.5% NaCl with a syringe into the adductor muscle through the predrilled hole. We monitored the survival of all animals daily and additionally collected 24 oysters distributed over all treatment groups on day 1, 3, and 5 post injection to extract haemolymph (100 µl) from the adductor muscle. We used 5 µl of the haemolymph to determine bacterial load expressed as colony forming units (CFU) by plating on TCBS agar plates.

Primary *in vitro* experiments

For the primary *in vitro* experiment, we used the haemolymph of the three groups of singly exposed oysters (hW, hT, hC see Fig. 1) isolated on day 1,3, and 5 in triplicate (Figure 1). Haemolymph was centrifuged at 10,000 x g at 25°C for 2 minutes in order to separate serum from the haemocytes. After centrifugation the supernatant serum was filter-sterilized to remove bacteria (0.22 µm pore size) and diluted 1:10 with nutrient medium. Serum from each experimental group (hT, hW, hC) from days 1, 3 and 5 was incubated with the two *V. splendidus* strains the sympatrically isolated O7w_July (vW) and the allopatrically isolated Tx5.1 (vT) which were cultured in a nutrient medium at 25°C for 24 h, and adjusted to 10^4 cells/µl (Wendling, C.C., and Wegner, K.M. 2015). Ninety-six well plates (Greiner Bio-One, Germany) were filled with 180 µl of diluted haemolymph serum and 20 µl bacteria solution, as well as negative control (culture medium only) and two positive controls *V. splendidus* strains: O7w_July (W) and Tx5.1(T) with only medium. In total we assayed 6 experimental groups (hT.vT, hW.vT, hC.vT, hT.vW, hW.vW, hC.vW) from three days (1,3, and 5 dpi) to give 18 experimental groups that we assayed in triplicates resulting in 54 samples. Growth curves were measured at 25 °C with shaking in a microplate reader (Synergy 2, Biotek) by taking measurements at OD_{550} nm every 15 minutes for 24 hours.

Secondary challenge: Bacteria preparation for infection and *in vivo* to cross- protection assay

Each of the primary challenge group (T, W, C see above) was divided into three groups with 8 oysters per group, which were then infected with sympatrically isolated *Vibrio* O7w_July, allopatrically isolated *Vibrio* Tx5 or nutrient solution 1.5% NaCl as control in a fully factorial design (Figure 1). We injected 100 µl of 10^8 cells/ml solution of *V. splendidus* strains T, W and equal volume of nutrient solution (1.5% NaCl) with a syringe into the adductor muscle through the predrilled hole. This resulted in nine experimental groups reflecting the combinations of primary and secondary challenges (primed with T: TT,TW,TC; primed with W: WT,WW,WC and control in primary challenge: CT, CW, CC). We collected 24 oysters distributed over all treatment group on day 1, 3, and 5 post injection to extract haemolymph (100 µl) from the adductor muscle. We used 5 µl of the haemolymph to determine bacterial load expressed as colony forming units (CFU) by plating on TCBS agar plates.

Secondary *in vitro* challenge to study cross- protection

The secondary in vitro cross-protection experiment followed a similar setup to the primary experiment. For the secondary *in vitro* cross protection experiment, the oyster haemolymph was divided into 9 groups for days 1 ,3 and 5 and kept in triplicates (Figure 3) resulting in nine groups of haemolymph (hTT: primed with T and exposed to T, hTW: primed with T and exposed to W, hTC: primed with T and exposed to control medium and accordingly for the other groups primed with W (hWT, hWW, hWC) or without priming (hCT, hCW, hCC), and 180 µl of 1:10 diluted haemolymph were added to a 96 well plate in triplicates. Cultures of both bacterial strains (W,T) were grown in nutrient medium at 25 C for 24 h, and 10^4 cells were added to each well (Wendling, C.C., and Wegner, K.M. 2015), an OD_{550} nm was measured every fifteen minutes for twenty-four hours to generate growth curves in a Synergy 2 plate reader (Biotek, Germany).

CHAPTER IV: The co-evolutionary context of immune priming and cross-protection in the oyster *Magalana (Crassostrea) gigas*

Primary Challenge

72 Pacific Oyster *Magalana (Crassostrea) gigas*

Divided to three groups (W, T and Control)

In vivo examination

Oysters exposed with 10^4 cell of *V. splendidus* T (Tx5.1, allopatric) and W (O7w-July, sympatric) strains

24 W 24 T 24 Control

Obtaining the oysters serums day 1,3,5 post infection

In Vitro examination

Examination in microplate reader ,Primary oysters serums day 1,3 and 5 post infection incubated 24 h with 10^5 cell of *vibro splendidus* sympatric, allopatric strains.

W T Control

Secondary challenge

In vivo examination

Oysters exposed with 10^7 cell of *V. splendidus* T (Tx5.1, allopatric) and W (O7w-July, sympatric) strains

8 WW 8 WT 8 WC 8 TT 8 TW 8 TC

8 CC 8 CT 8 CW

Obtaining the Oysters serums day 1,3,5 post infection

In Vitro examination

Examination in microplate reader , oysters serums day 1,3 and 5 post infection incubated 24 h with 10^5 cell of *vibro splendidus* sympatric, allopatric strains.

WW WT WC TT T W TC

CC CT CW

Figure 1:

The depicted figure delineates primary and secondary challenges within a sequence of both in vivo and in vitro experiments conducted on Pacific oysters *Magalana (Crassostrea) gigas*. In the primary in vivo phase, oysters were subjected to infection with *Vibrio splendidus* strains sympatric or W (O7w_July) and allopatric or T(Tx5.1).

During the primary in vitro experiments, haemolymph obtained from individually exposed oysters served as a pivotal component for evaluating the immune response. The haemolymph serum underwent meticulous filter-sterilization and subsequent incubation with *V. splendidus* strains with a microplate reader, followed by the measurement of resulting growth curves over 24 hours.

Subsequently, in the secondary challenge, oysters involved the division of primary challenge groups into three subsets. These subsets were subjected to infection with either sympatric or allopatric *Vibrio* strains within the treatment groups, while the control group received a nutrient medium. Haemolymph was systematically collected once more to ascertain bacterial load.The secondary in vitro challenge, designed to study cross-protection, is carried on the experimental setup of the primary experiment. Haemolymph was categorized into nine distinct groups and strategically placed within a 96-well plate. Bacterial strains were introduced, and ensuing growth curves were meticulously monitored

 represents a Pacific oyster *Magalana* (*Crassostrea gigas*).

 represents a microplate icon for in vitro challenges.

 represents icon for oyster's serum.

Statistical analysis

All data were analyzed with R version 4.1.3. We analyzed *in vivo* CFU counts using the GLM function with both a Poisson, quasi-Poisson and a negative binomial error distribution and used AIC model comparison to find the best fitting model with, day bacterial strain and their interaction as fixed factors. The best-normalize package was used to transform data from the *in vitro* experiments for use in linear models using the Ordered Quantile transformation. For *in vitro* data AIC model comparison was carried out to find the best fitting combination of fixed factors and interactions (Tables S1 and S8). For bacterial growth Gompertz curves were fitted to the growth rate data using the *growthcurve* package and carrying capacities (K) were calculated from these

curves. Differences were deemed significant at $p < 0.05$, If significant differences were indicated at the 0.05 level, then post hoc multiple-comparison (Tukey's) tests were carried out using the 'multcomp' and 'esmeans' packages in R to examine significant differences among treatments.

Results

Primary challenge

Bacterial load *In vivo* For the colony-forming units (CFUs) counts from plated out haemolymph high Cook's distances were found in the poisson and quasipoisson models. AIC comparison of the poisson and negative binomial model showed that the negative binomal model was the better fit (poisson 782, negative binomial 246). Using this model, CFU counts differed significantly by the day of haemolymph extraction post injection (Negative binomial GLM: Chisq = 13.2, d.f. = 2, p=0.001, Table S4), the strain of bacteria the host was inoculated with (Negative binomial GLM: Chisq = 38.3, d.f. = 2, p<0.0001) and the interaction of the two (Chisq = 12, d.f. = 4, p=0.017). The posthoc multiple comparison tests revealed that injection with sympatric *Vibro* W resulted in higher bacterial loads than either allopatric bacteria or control inoculation and injection with allopatric bacteria resulted in higher bacteria loads than control treatment only on day 5 (comparision of means, p<0.05, Figure 2, Table S5). Over time, bacterial load significantly decreased only in the control treatment (pairwise comparison of means, $p < 0.05$). However, there was a trend towards a decrease in bacterial load in the sympatric treatment (pairwise comparison of means, $p = 0.09$).

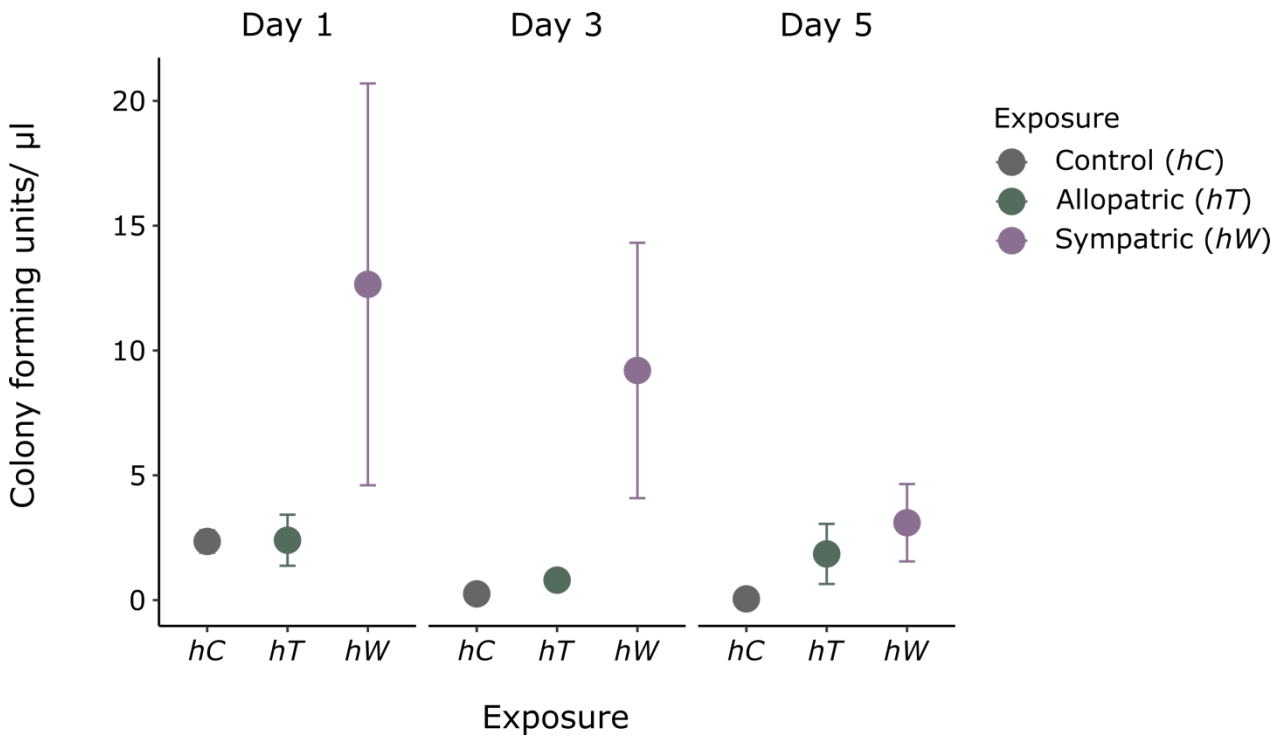

Figure 2. *In vivo* experiment priming in the two infection groups W *(* infected by the sympatric *Vibrio* O7w_July) and group T (infected by the allopatric *Vibrio* Tx5.1 as well as the control group of oysters on day 1,3 and 5 post infection. Challenge with sympatric *Vibrio vW* resulted in higher bacterial loads than allopatric *Vibrio* or control inoculation and injection with allopatric bacteria resulted in higher bacteria loads than the control treatment only on day 5.

Bacterial growth with haemolymph *in vitro*

Carrying capacities of hemolymph from the oysters used for the priming exposure were determined for both sympatric OW7_july (W) and Tx5.1 (T) bacteria. For the primary exposure the data were transformed using an ordered quantile normalizing transformation to achieve the most accurate normal distribution. We used AIC model selection to compare a range of models including day, the bacteria the oysters were injected with (W or T) and the bacteria grown with exposed haemolymph *in vitro* (W or T) to find the best fitting model (Table S1). The model that best fit the data contained all three terms and the interaction between all three with an Akaike weight representing a relative likelihood of 84% (Table S1). There was a significant difference in bacterial growth rate in vitro among injection treatments (linear model: $F = 13.57$, d.f. =2, $p < 0.0001$, Figure 3, Table S2). The growth in the two *Vibrio* treatments did not differ significantly from one another, however, bacteria reached higher carrying capacities in both in comparison to the control (Tukey multiple comparison

tests $p<0.05$, Figure 3, Table S3), suggesting that here may have been substances in the haemolymph that promote growth. Both vW and vT grew best in the haemolymph challenged with the matching strain (vW in hW and vT in hT), indicating that there was some strain specific serum alteration to favor growth of the matching strain. This effect was strongest for sympatric vW indicating that vW might have evolved mechanisms to maximise habitat manipulation in the oyster tissue. Interestingly, however, bacterial carrying capacity *in vitro* is significantly reduced over time. Again, this effect was strongest when oysters were injected with sympatric *vW* (Tukey multiple comparison test, difference between days one and five, $p<0.05$, Figure 3, Table S3). This is the opposite to the pattern seen in control injected oysters, where the carrying capacity on day one is lower but increases significantly with allopatric bacteria (Tukey test $p <0.05$, Figure 3, Table S3) and near significantly (Tukey test $p=0.065$, Figure 3, Table S3) with sympatric bacteria. This might indicate that the most efficient inhibition response is mounted against sympatric vW, and that the dynamics of the balance between growth enhancement and inhibition is dependent on shared evolutionary or environmental history.

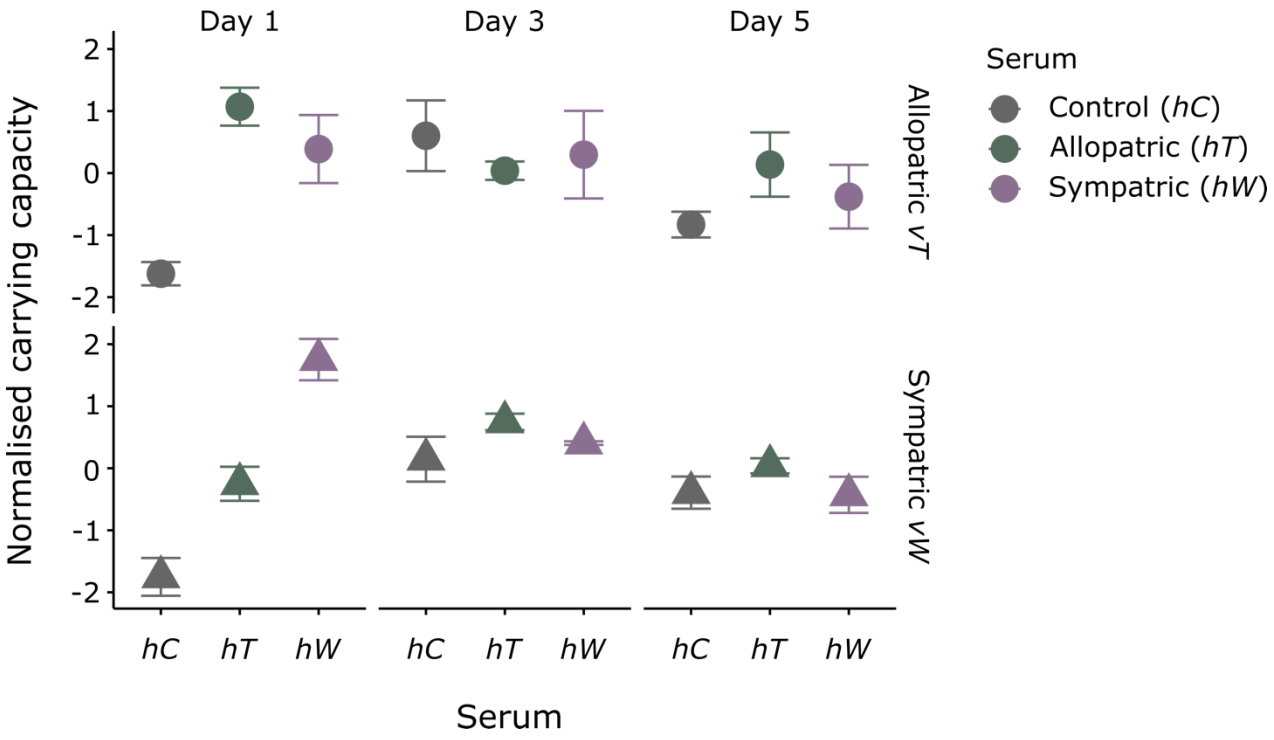

Figure 3: Carrying capacities of hemolymph serum primed with different exposures (hW, hC and hT) for *Vibrio* strains vT and vW after 1,3 and 5 days post infection . Primary exposure with either strain increased carrying capacity, but was strongest for matching combination of Vibrio strain and hemolymph on day 1 (vW – hW, vT-hT). Highest carrying capacity was reached for the vW-hW combination of day 1, but decreased significantly over time, whereas low carrying capacities were only found in hC on day one, but also increased significantly with allopatric bacteria (Tukey test<0.05), and showed a trend with sympatric bacteria (Tukey test p=0.065) throughout the course of the experiment.

Secondary challenge

Bacterial load in vivo

The other objective was to determine whether initial priming with a low dose of either sympatrically or allopatrically isolated bacteria, resulted in a specific response to a second higher dose (10^7 cells) of either the sympatric or allopatric bacterial strain. Here, the negative binomal model was the best fit with a lower AIC than the poisson model (poisson: 8617, negative binomial 911) and a lower deviance than the quasipoisson model. Primary strain (Negative binomial GLM: Chisq = 6.01, d.f.

= 2, p=0.05, Table S6) significantly influenced the number of bacterial cells accumulating *in vivo*, with CFUs being significantly higher when primed with allopatrically isolated *Vibro* (vT) than in the control treatment (comparison of means, $p<0.05$, Figure 4, Table S7). Secondary strain also significantly influenced the outcome (Negative binomial GLM: Chisq = 8.36, d.f.=2, p=0.015, Table S6), which was again a result of individuals infected with allopatrically isolated bacteria (vT) having higher bacterial loads than the control (comparison of means $p<0.05$, Figure 3, Table S7). The interaction between primary and secondary exposure was also significant (negative binomial GLM: Chisq = 11.2, d.f. = 4, p = 0.024, Table S6), indicating that priming influences the response to a later exposure. Interestingly, bacterial load was significantly higher in oysters exposed twice to both allopatric bacteria than when primed with either sympatric bacteria or control injected and also higher when exposed twice to sympatric bacteria than a control infection but not allopatric priming (pairwise comparisons of means, $p<0.05$, Figure 6, Table S7). These data suggest that priming leads to increased *in vivo* growth, rather than inhibition and mirror the first set of *in vitro* results where we saw higher carrying capacities with homologous combinations of primed hemolymph and infecting strain.

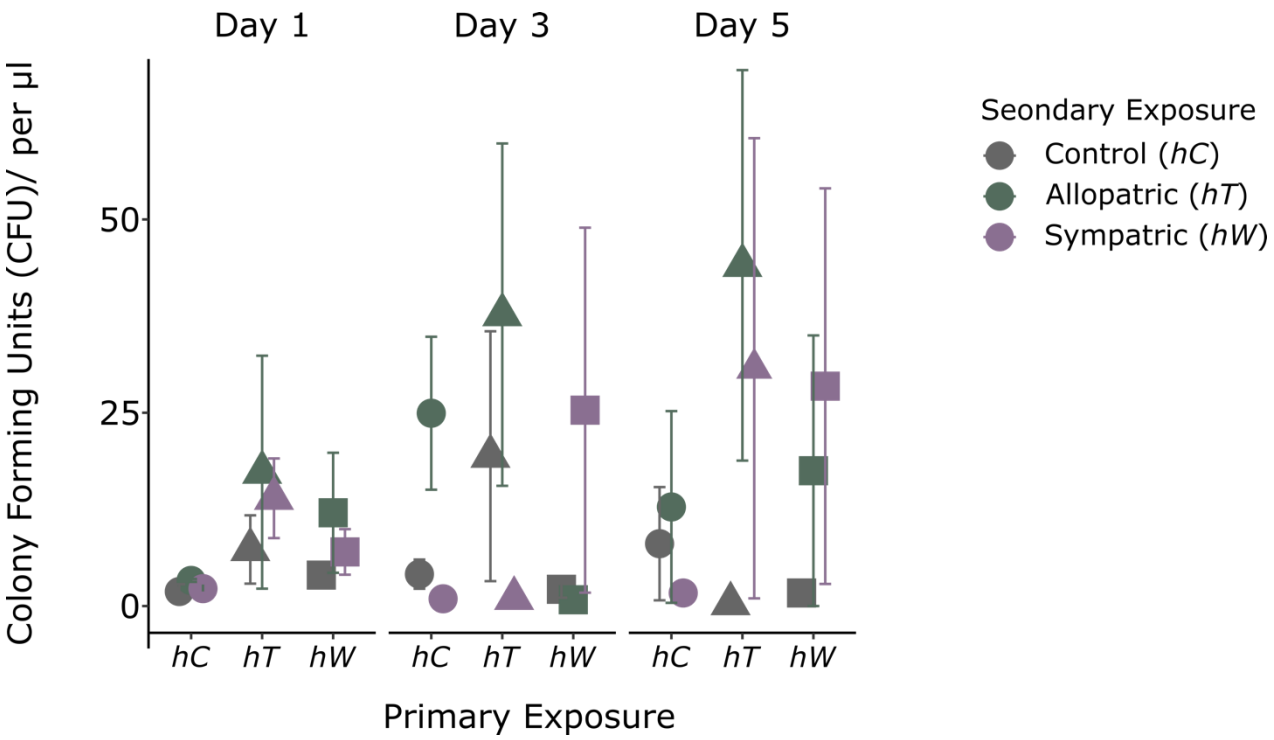

Figure 4. Bacterial load after the secondary challenge of oysters primed with sympatric (vW) and allopatric (vT) *V. splendidus.* A significant interaction between the primary and secondary exposures in the *in vivo* experiment (Negative binomial GLM: Chisq = 11.2, d.f. = 4, p = 0.024) indicates that priming influences the response to subsequent exposures. Oysters primed with sympatric or allopatric bacteria showed significantly higher bacterial loads when exposed twice, suggesting priming increases in vivo growth rather than inhibiting it.

Inhibition after secondary challenge *in vitro*

The *Vibrio* growth data, on the other hand, suggested a degree of inhibition when bacteria were grown in serum from doubly exposed oysters in vitro. Data were transformed using an ordered quantile normalizing transformation, and the best fitting model contained the fixed factors primary and secondary exposure and the interaction of the two (100% Akaike weight, Table S8). The effect of primary treatment alone was marginally non-significant (Nested linear model: Chisq = 5.3, d.f.= 2, p = 0.07), however, secondary treatment had a significant effect (Nested linear model: Chisq = 103.83, d.f.= 2, p < 0.0001, Figure 5), with treatments with both bacterial strains resulting in significantly lower carrying capacities than the control treatment (Tukey multiple comparison tests p<0.05, Table S9). The interaction between primary and secondary treatment was also significant

(Nested linear model: Chisq = 67.4, d.f.= 4, $p < 0.0001$). Within the control secondary treatment there were no significant differences among primary treatments. Within the allopatric secondary treatment, both primary treatment with allopatric and sympatric bacteria resulted in significantly higher carrying capacities than the control (Tukey multiple comparison tests $p<0.05$, Figure 5, Table S9) but did not differ from one another. Within the sympatric secondary exposure, however, carrying capacities were significantly lower in the sympatric primary treatment than in either the control or allopatric treatments (Tukey multiple comparison tests $p<0.05$, Figure 5, Table S9), whereas the control and allopatric treatments did not differ from one another. In contrast to the primary exposure, the hemolymph exposed to sympatric vW twice showed the biggest inhibitory effect, suggesting that some specific immune memory against the locally encountered sympatric strain could have evolved.

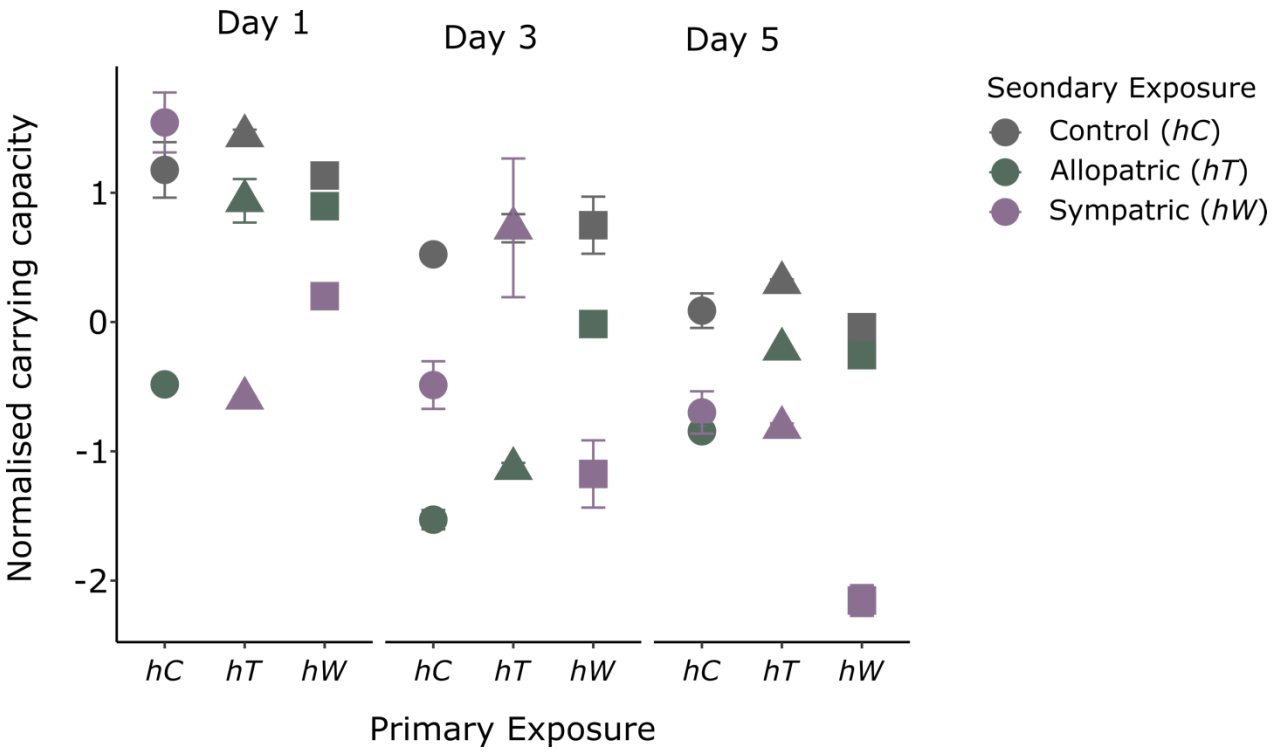

Figure 5. Carrying capacities of hemolymph serum after priming and secondary exposure with *Vibrio* strains vT and vW after 1,3 and 5 days post infection data from growth with vT and vW were pooled for simplification as the strain being grown hat no significant impact on the outcome and was exclused in the best fitting model. **T**he interaction between primary and secondary infection was significant (Nested linear model: Chisq = 67.4, d.f.= 4, $p < 0.0001$). Within the control secondary treatment there were no significant differences among primary treatments. Within the allopatric secondary treatment, both primary treatment with allopatric and sympatric bacteria resulted in significantly higher carrying capacities than the control (Tukey multiple comparison tests $p<0.05$) but did not differ from one another.

Discussion

We investigated *in vitro* and *in vivo* immune priming with sympatric and allopatric strains of *V. splendidus* in Pacific oysters. We specifically asked whether priming with sympatric bacteria, sharing evolutionary and environmental history with their host, differs from priming with allopatric bacteria. We observed the growth dynamics of bacteria both *in vivo* and *in vitro*. We found that sympatric *Vibrios* seem to be better adapted to colonizing oysters than allopatric *Vibrios*. Yet the

oyster haemolymph, when oysters are immune primed with the sympatric *Vibrios*, shows the highest inhibitory effects *in vitro*. The latter lends strong support to the notion that immune priming against bacterial infections occurs in *M. gigas*, although *in vivo* we do not see a similar effect. As the colonization in the primed host individuals is very high, this result is almost certainly driven by the colonization from the primary treatment. So far in oysters, immune priming has mainly been described for viral infections (Lafont *et al.*, 2020). We show here that cell free haemolymph serum of oysters primed with allopatriacally isolated *Vibrio* has inhibitory properties against the growth of *Vibrio* bacteria when subsequently challenged. This strongly supports previous immune expression data (Zhang *et al.*, 2014) in demonstrating priming in the *M. gigas* – *Vibro* system.

While the *in vitro* results are consistent with the hypothesis of immune priming, our *in vivo* results indicate that priming, contrary to our initial expectations, leads to an increase in *in vivo* bacterial growth rather than inhibition. This suggests that the initial exposure to the bacteria enhances their growth within the host organism. However, in contrast to the *in vivo* findings, the *in vitro* experiments revealed a degree of inhibition upon a second exposure to *Vibrio*. Specifically, when the bacteria were examined in haemolymph obtained from doubly exposed oysters, their growth was somewhat suppressed. There are several reasons that might potentially contribute to this incongruity. The changes in immune response upon priming with *Vibrio* reported here could be influenced by both cellular and humeral immune components (Wang, L.L *et al.*, 2018 REFs). Here our *in vivo* data encompass the potential for both factors to be at play. On the other hand, in our *in vitro* experiments we use cell-free filtered hemolymph or serum. This means that priming expressed in the form of cellular immunity, for example, phagocytosis would not influence the experimental outcomes. In effect, in the *in vitro* experiments we purely test the impact of priming via humeral immune defenses. In the context of *in vivo* conditions, oysters may experience additional regulatory variables or cellular interactions that influence their immune responses in a manner distinct from what is observed in isolated in vitro environments.

It is interesting to note that treatments involving both bacterial strains (allopatric and sympatric) showed a significant increase in carrying capacities compared to the control treatment. We also observed a significant interaction between the primary and secondary treatments, indicating that their combined effects on growth are not merely additive but rather exhibit an interactive

relationship. Intriguingly, no significant differences were observed between the primary treatments within the secondary control treatment. This implies that the specific type of primary treatment did not have a significant impact on growth when no secondary exposure occurs.

Our data support the hypothesis that the immune response of *M. gigas* is altered after inoculation with heat killed or sublethal doses of *Vibrio*. Upon initial contact, oysters rapidly identify *Vibrio* bacteria by recognizing certain molecular patterns associated with these bacteria (Huang, Q.S., *et al* ,2018). This initiates a series of immunological responses, which involve the activation of immune cells and the synthesis of antimicrobial substances. The immune system of oysters acquires the ability to distinguish several species of *Vibrio*, resulting in enhanced efficiency and accuracy of immune responses (Wendling, C.C., and Wegner, K.M. 2015), providing the potential for specificity in primed responses. Multiple exposures to *Vibrio* bacteria result in an augmented and specific immune response (Fallet, M., Montagnani ,*et al* 2022), which confers durable immunity against subsequent Vibrio infections. The acquired immunity demonstrated by oysters may persist for an extended duration, hence reducing their susceptibility to infections triggered by *Vibrio* bacteria. In this study, secondary challenge in vitro experiment demonstrated a sharp decrease in caring capacity in both strains when combined with sympatric-sympatric haemolymph. This suggests of a specific immune activation by the sympatric strain that elicits broad effectors working against both closely related strains.

Invertebrates in general have been the subject of a long discussion regarding their immunological memory, and several reports have suggested that some forms of immune memory may exist in invertebrates, generally referred to as specific immune priming (Schmitt, P.,*et al*,2013). Most studies investigating immune priming focus on enhanced survival due to changes in immune responses (Rowley, A.F., Powell, A., 2007) following previous encounter with pathogens or their products, providing protection against reinfection (Contreras Garduño, J. *et al*,2016).

For example, Insects, which were previously believed to lack immunological memory owing to that they do not posses specific memory cells, are now known to illict immune priming (Prakash and Khan, 2022). This particular method has the potential to undergo fast evolution in response to different circumstances (Prakash A, Khan ,2022). Certain insects exhibit a phenomenon referred to immune priming, wherein their immune response is enhanced after being exposed to a sublethal

dosage of a pathogen or pathogen-derived substance. This heightened immune response then provides resistance to a subsequent lethal infection within a short period of time (Sheehan G, *et al.*, 2020). These studies investigate a paradigm shift in the comprehension of insect immune responses, with particular emphasis on the identification of immune priming. Recent research indicates that immune priming, which protects against subsequent pathogen infections, can be induced by exposure to low levels of infection, in contrast to the conventional notion that insects lack immunological memory. This phenomenon presents a challenge to established beliefs and suggests that insect immune systems are more sophisticated and adaptable. The capacity for swift adaptation in light of varying conditions introduces intricacy to this recently acquired comprehension.

In summary, this study underscores the multifaceted dynamics of bacterial priming on oyster populations, with implications for understanding the intricate interplay between bacterial strains, priming sequences, and their impact on humeral and cellular immune response ultimately determining oyster health and bacterial growth. We demonstrated that immunopriming stimulates oyster immune responses against both sympatric and allopatric strains of *V.splendidus* in order to enhance specific memory and adaptive immunity in oysters. This knowledge could be used for vaccine programs to prevent disease outbreaks in aquaculture.

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CHAPTER V: GENERAL DISCUSSION

In this thesis, I involved an analysis of three offered hypotheses that attempt to offer an explanation for the phenomena being investigated. The current study aims to examine the antibacterial characteristics of extracts derived from marine sea cucumbers and oysters in relation to their effectiveness against *Staphylococcus aureus* and *Vibrio* bacteria.

The first hypothesis I studied predicts the potential antibacterial effects on methicillin-resistant *Staphylococcus aureus* (MRSA) and enterotoxin-producing *Staphylococcus aureus* strains (SEASA, SEBSA) by the utilization of bioactive component extracted from the sea cucumber *Holothuria leucospilota.* The investigation conducted on bioactive substances obtained from sea cucumbers in the Persian Gulf revealed their potential as antibacterial agents against MRSA and SEBSA strains. The extracts, obtained by the use of chloroform or methanol, had inhibitory effects on growth of all strains tested, with minimum inhibitory concentrations (MIC) reaching up to 100 mg/ml. The methanol extract had the strongest antibacterial action (chapter II).

A suitable model for investigating the dynamics of *Vibrio* and host interactions in illness is the utilization of *Crassostrea gigas* in the North Sea, which allows for the examination of invasive sources of infections, particularly *Vibrio kanaloae*. In this investigation, molecular fluorescent in situ hybridization (FISH) techniques were employed to promptly determine the bacterial diversity inside the oyster species *Crassostrea gigas* originating from Sylt Island. Additionally, the identification of all strains was confirmed by the validation of their 16SrRNA gene sequences. Oysters were collected and subjected to exposure with strains T02 and S12 of *vibrio kanaloae*. Subsequently, various tissues were investigated using culture-independent techniques. The digestive glands, gill and Muscle in *vibrio kanaloae* were identified as metabolically active by FISH technique, one of the advantages of the FISH technique is that it is accurate and very easy to use. This technique allows rapid assessment of *Vibrio* in oysters. The study aimed to detect *Vibrio kanaloae* bacteria colonization in *Crassostrea gigas* oysters from Sylt Island using fluorescent in situ hybridization (FISH) and 16SrRNA gene sequencing. The research found that *Vibrio* strains were

detected in digestive glands, gills, and muscle tissues three days post-infection. The study found that both strains had a few colonization in gill tissue and similar amounts in digestive glands. However, the detection of these strains in muscle tissue was rare or minimal. The findings highlight the importance of FISH in evaluating *Vibrio* populations in oysters. (chapter III).

I also studied on *Magalana gigas* oysters are at risk of infection caused by pathogenic *Vibrio* strains, leading to significant impacts on worldwide output. In the context of our investigation into the susceptibility of *Magalana gigas* oysters to pathogenic *Vibrio* strains, a crucial aspect of our study involved delving into the phenomenon of priming. Priming, in the immune response of oysters, refers to the process by which the immune system is sensitized and prepared for a more robust reaction upon subsequent exposure to the same or related pathogens.

In this study, we selected two closely related *Vibrio splendidus* strains to specifically explore the dynamics of priming. The objective was to examine how the oysters' immune system responds to initial exposure and whether it exhibits an enhanced defense mechanism upon subsequent encounters. Our findings revealed that when the oysters were exposed to 10^4 bacterial cells, notable inhibitory effects were observed during successive challenges. Furthermore, a compelling observation emerged from the analysis of the oysters' haemolymph during the secondary challenge. The haemolymph, a key component of the oysters' circulatory system, exhibited significant inhibitory effects on the sympatric *V. splendidus.* This indicates a systemic response within the oysters, suggesting a heightened immune reaction following the initial exposure.

The significance of matching local strains becomes apparent in this context. The process of selecting closely related *Vibrio splendidus* strains for exposure is not merely an experimental choice but has immunological implications. Matching local strains have the potential to activate specific immune responses, a phenomenon commonly associated with priming. This activation, in turn, provides a level of protection against vibriosis in vivo, as demonstrated in the findings presented in Chapter IV of this dissertation. In summary, our study not only sheds light on the susceptibility of *Magalana gigas* oysters to pathogenic *Vibrio* strains but also underscores the importance of priming in the oysters' immune response. The intricate interplay between exposure, immune activation, and subsequent protection has significant implications for understanding and mitigating the impacts of vibriosis on oyster populations worldwide.

GENERAL DISCUSSION

Anti-bacterial effect of Sea cucumber *H. leucospilota* extract

The prevalence of seafood-borne food poisoning caused by the preformed enterotoxins of Staphylococcus aureus is a global concern. In this context, aquatic organisms, including those consumed as seafood, have gained attention not only as potential vectors of foodborne pathogens but also as sources of bioactive compounds with significant medical implications. Among these organisms, the sea cucumber *Holothuria leucospilota*, prevalent in the Persian Gulf, has emerged as a subject of interest in the exploration of antimicrobial activities.The sea cucumber, *Holothuria leucospilota*, has been a subject of extensive research due to its unique biological properties and the potential therapeutic value of its bioactive compounds (Ridzwan *et al*., 1995).Numerous studies have explored the diverse array of chemical compounds present in sea cucumbers, ranging from triterpene glycosides to peptides, with documented antimicrobial, anti-inflammatory, and antioxidant properties(Yasoda *et al*., 2006; Mohammadizadeh *et al*., 2013; Kiani *et al*., 2014). The rich biochemical profile of sea cucumbers has made them a focal point in biomedical research, aiming to harness their natural compounds for pharmaceutical and therapeutic applications.

In the specific context of our study, we focused on extracting bioactive compounds from whole sea cucumber bodies using chloroform or methanol. This extraction process aimed to capture a spectrum of compounds with potential antimicrobial properties. The choice of solvents, chloroform, and methanol, allowed for the extraction of different classes of compounds, enriching our understanding of the sea cucumber's antimicrobial repertoire. we delved into investigating the antimicrobial activity of the extracted compounds against *Staphylococcus aureus*, a common agent of seafood-related food poisoning. The primary objective this investigation was to evaluate the sea cucumber's potential role as a source of antimicrobial agents that could combat or inhibit the growth of pathogenic bacteria. Microorganisms utilize many strategies to mitigate the concentration of antibacterial agents (Mokhlesi *et al*., 2011) .

The examination of either the body walls or the entire bodies was conducted in the majority of the sea cucumber species investigated (Ridzwan *et al*., 1995; Yasoda *et al*., 2006; Mohammadizadeh *et al*., 2013; Kiani *et al*., 2014). Furthermore, Adibpour *et al.* (2014) have demonstrated that

holothurian chemicals exhibit antibacterial action, although at much elevated doses. It is important to highlight that alternative species or populations of sea cucumber may exhibit varying degrees of activity in comparison to those seen in our study. on a study conducted by Yasoda *et al* (2006), the researchers examined the effects of a methanol-acetone extract derived from the body wall of *Parastichopus parvimensis*, a species of sea cucumber found on Santa Catalina Island, California. The extract was tested for its antimicrobial activity against *Escherichia coli* using the disc diffusion method. The study included two antibiotics, Ampicillin and Tetracycline, as positive controls. The antimicrobial efficacy of the extract against *Escherichia coli* has been validated. However, the extract's zone of inhibition was significantly narrower compared to that of the antibiotics. Furthermore, Farjami *et al*.(2013) observed modest bacteriostatic efficacy against a limited number of Gram-negative and Gram-positive bacterial species while conducting experiments using methanol and chloroform extracts obtained from different tissues and organs of *H. leucospilota*. However, the study conducted by Adibpour *et al*. (2014) did not observe significant bacteriostatic effects against *Shigella dysenteriae, Proteus vulgaris, Bacillus cereus, S. epidermidis*, and *Candida albicans* at the dosages tested. Previous research has posited that the active molecule in question is a lysozyme, as indicated by Gaidi *et al*. (2001).

The antimicrobial activity of the whole-body extracts of *H. leucospilota* observed in our study, specifically against MRSA, SEASA, and SEBSA. These activities may be attributed to many factors, which warrant further investigation. Hence, it is essential to conduct thorough chemical investigations in order to isolate and purify the active compounds, ascertain their chemical composition, and assess their potential appropriateness as novel therapeutic candidates (Adibpour *et al.,* 2014). In their study, Mokhlesi *et al*. (2011) analyzed extracts obtained from the body wall of sea cucumbers sourced from the Persian Gulf. The extracts, including methanol, ethyl acetate, and water-methanol, were evaluated for their effects on three bacterial strains: *Staphylococcus aureus, Escherichia coli*, and *Pseudomonas aeruginosa.* However, the researchers saw no discernible inhibitory impact of the extracts on bacterial growth. In their study, Farjami *et al*. (2013)investigated the antibacterial properties of hexane, chloroform, and methanol extracts derived from the body wall, gonad, and gut of *H. leucospilota*. The extracts were tested against three bacterial strains, namely *S. aureus*, *Bacillus subtilis*, and *Pseudomonas aeruginosa* has the highest susceptibility to the extracts, since no antibacterial activity is observed at any dose against *Bacillus subtilis* and *Staphylococcus aureus*. The factors that account for the disparities between

our findings and the extant body of research remain unclear at present. Additional factors that may contribute to the observed variations include variations in extraction methodologies, variations in the types of solvents employed, and variations in the ecological circumstances of the specimens under investigation. The quantity of samples and extracts used in this investigation is a point of concern. Despite the fact that we only possess three strains of *Staphylococcus aureus* and two collected extracts (one with chloroforms and one with methanol), these strains are extremely important and are utilized in related research. In addition, although the collected number of samples prevents a comprehensive statistical analysis of sea cucumber variation, it should be sufficient to provide an adequate comprehension of the species in general. However, for comprehensive statistical analysis, we recommend that future studies involve a greater number of samples and extracts. My thesis showed that sea cucumber extract, derived from *H. leucospilota* from the Persian Gulf, could be used to prevent bacterial contamination in seafood. Sea cucumbers are known for their unique bioactive properties, and their extracts have antibacterial properties against S. aureus strains. However, further research is needed to understand their chemical makeup and mechanisms of action. Additionally, cultivating sea cucumbers in aquaculture could provide sustainable bioactive compounds for the pharmaceutical and seafood industries.

Outlook

The project's perspective entails the necessity for thorough research aimed at the isolation and characterization of the bioactive components found in *Holothuria leucospilota* sourced from the Persian Gulf. Additional investigation is imperative in order to separate and delineate these active constituents and ascertain their potential as agents that resist microorganisms. Furthermore, it is imperative to carry out in vivo and clinical investigations in order to evaluate the safety and effectiveness of these compounds in various medical and food safety contexts.

Identification of *Vibrio kanaloae* in the pacific Oyster *Crassostrea gigas* by Fluorescence in situ Hybridization

The main goal of this research endeavor was to identify the presence of *Vibrio kanaloae* bacterial colonization in *Crassostrea gigas*, a species of oyster native to Sylt Island. This was studied using

fluorescent in situ hybridization (FISH) methodology, which was subsequently supplemented with 16SrRNA gene sequencing to validate the identification. The research focused on investigating the identification of *Vibrio kanaloae* strains T02 and S12 in various tissues of oysters, including the digestive gland, gill, and muscle, at the three-day post-infection. This research utilized methods that were not influenced by cultural factors. The utilization of Fluorescence In Situ Hybridization (FISH) demonstrated successful identification of *Vibrio kanaloae* strains T02 and S12 within the muscular tissues, digestive gland, and gills of *Crassostrea gigas*. The implementation of this technique enabled a rapid examination of the *Vibrio* species present in the oyster as a result of its exceptional specificity and user-friendly characteristics. The investigation demonstrated obvious patterns of *V. kanaloae* colonization in various oyster tissues. *Vibrio* strains S12 and T02 were both detected in the gill tissue, although in a few amounts. Few bacteria were detected in the digestive tissues, which contained comparable quantities of both *Vibrio* strains. It is noteworthy that the observation of *V. kanaloae* strains T02 and S12 in the muscle tissue of oysters was rarely or invisible, suggesting a possibility of a feasible difference in their temporal distribution within the organism. In recent years, there has been a rise in the consumption of aquatic products, which has prompted public health concerns regarding the need for more accurate, rapid, and sensitive pathogen detection.

The use of aquatic products has witnessed a notable surge in recent years, hence giving rise to public health issues that need the development of more efficient and accurate pathogen detection methods, characterized by enhanced sensitivity, speed, and specificity (Arfatahery, N *et al.*, 2016).Presently, culture methods serve as the primary means of identification for *Vibrio spp*. This methodology is characterized by a significant investment of time, often spanning a duration of four to seven days, in order to obtain definitive outcomes. Molecular approaches have demonstrated their efficacy in facilitating expedited and accurate pathogen detection in aquatic items, including *Vibrio* strains (Panicker, G , 2005; Messelhäusser, U *et al.*, 2010 ; Paranjpye, R *et al.*, 2012 ; Tall, A *et al* , 2012). Polymerase chain reaction (PCR), a fundamental molecular method, is widely employed for the identification of bacteria (Fakruddin, M.D *et al.*, 2013; Avendaño-Herrera, R *et al.*, 2014). The utilization of DNA microarrays for the purpose of diagnosing *Vibrio* has also been documented in previous studies (Kim, I.H.,*et al.*, 2011 ; Lee, Chae-Yoon , 2011).

In the context of our research on *Vibrio*-host interactions in *Crassostrea gigas* and the utilization of molecular fluorescent in situ hybridization (FISH) methods, it is worth noting that FISH has been utilized in a multitude of studies to examine the dynamics of *Vibrio* species within these aquatic organisms. These investigations have yielded significant knowledge regarding the abundance, dispersion, and functionality of pathogenic bacteria. The utilization of FISH, which enables the direct visualization of particular bacterial clusters within tissues, has been crucial in elucidating the complex interconnections that exist between *Vibrio* species and their molluscan hosts.Our study incorporated the validation of FISH results through 16SrRNA gene sequencing, aligning with contemporary study(Ogayar E *et al.*, 2021)) emphasizing the importance of combining molecular techniques for robust microbial identification. This dual approach enhances the reliability of microbial community assessments, ensuring accurate taxonomic assignments and contributing to a comprehensive understanding of the microbial landscape within oysters.We studied tissues of oysters on day 3 post-infection, The infrequent and limited prevalence of *V. kanaloae* strains in the muscular tissue of oysters revealed in our study suggests a potential temporal discrepancy in their distribution. The variation in tissue colonization patterns over time emphasizes the need for longitudinal studies to capture the dynamic nature of host-pathogen interactions.

Outlook

The research viewpoint entails the broadening of the spectrum of investigations concerning the interactions between *Vibrio* and oyster hosts, with a specific focus on exploring the potential implications for the management of diseases. This study has the potential to contribute to the advancement of more precise approaches in the prevention and management of Vibrio-associated illnesses within oyster communities.

In vitro* and *in vivo* evaluation of immune priming and Cross- protection in the oyster *Magalana (Crassostrea) gigas

In this study, we examined the immunological priming effects of sympatric and allopatric strains of *V. splendidus* on Pacific oysters, both in vitro and in vivo. We specifically inquired about the potential differences between priming with sympatric bacteria and priming with allopatric bacteria.

GENERAL DISCUSSION

The growth dynamics of bacteria were studied in both in vivo and in vitro settings. Our research indicates that sympatric *Vibrios* exhibit a higher degree of adaptation to oyster colonization compared to allopatric *Vibrios*. However, the haemolymph of oysters has significant inhibitory effects in vitro when oysters are immunologically primed with sympatric *Vibrios*.The latter provides substantial evidence in support of the concept that immunological priming against bacterial infections takes place in *M. gigas*. However, this impact is not observed in vivo. The observed high colonization in the primed host individuals is probably attributable to the colonization resulting from the first treatment. To date, the phenomenon of immune priming in oysters has been specifically documented just in the context of viral infections (Lafont *et al*., 2020). In this study, we demonstrate that the haemolymph serum of oysters, which have been exposed to allopathically isolated *Vibri*o, has inhibitory characteristics against *Vibrio* bacteria upon subsequent challenge. The findings of this study provide robust evidence that aligns with the previous immunological expression data offered by Zhang *et al*. (2014), hence reinforcing the concept of priming within the *M. gigas* - *Vibro* system.

The findings obtained from our *in vitro* experiments align with the idea of immune priming. However, our in vivo experiments reveal a contrasting outcome, as they demonstrate that priming actually results in an elevation of *in vivo* bacterial growth, rather than its suppression, which contradicts our initial predictions. This observation implies that the initial contact with the bacteria amplifies their proliferation within the host organism. However, in contrast to the findings shown in living organisms, the trials conducted in a controlled laboratory setting demonstrated a certain level of inhibition following a subsequent encounter with *Vibrio*. In particular, the development of the bacteria was seen to be somewhat inhibited when they were analyzed in haemolymph derived from oysters that had been exposed to both factors simultaneously. There exist other factors that might potentially lead to this incongruity. The alterations in immune response following exposure to *Vibrio*, as described in this study, may be impacted by both cellular and humoral immunological factors (Thompson FL,*et al* , 2004; Lee KA, ,*et al* , 2004 ; Cray, C,*et al*,2007 ;Tao Zhang,*et al* , 2014; Eckersall, P.D *et al*,2010) Our in vivo data includes the possibility for both elements to be involved. In contrast, our *in vitro* research involve the use of cell-free filtered haemolymph or serum. This implies that the activation of cellular immunity, such as phagocytosis, would not have an impact on the results of the experiment. In the present study, we only examine the effects of priming through humoral immune defenses in in vitro trials. Under in vivo situations, oysters may

encounter supplementary regulatory factors or cellular interactions that exert an impact on their immune responses in a manner that differs from what is found in isolated in vitro environments.

Treatments including both bacterial strains (allopatric and sympatric) demonstrated a considerable increase in carrying capacities when compared to the control treatment. This shows that when both strains are present together, they have a synergistic effect on bacterial growth. We also found a significant interaction between the primary and secondary treatments, demonstrating that their combined effects on growth are not only additive but also interact. Interestingly, no statistically significant changes were found between the primary treatments and the secondary control treatment. This implies that the specific type of primary treatment did not have a significant impact on growth when no secondary exposure occurs

The findings presented in this study provide evidence in favor of the concept that the immune response of *M. gigas* undergoes modifications following inoculation with heat-killed or sub lethal concentrations of *Vibrio*. During their first contact, oysters promptly detect *Vibrio* bacteria by recognizing certain molecular patterns that are linked to these bacteria (Tao Zhang *et al.*, 2004). This triggers a cascade of immunological reactions, encompassing the stimulation of immune cells and the production of antimicrobial compounds. The immune system of oysters develops the capacity to differentiate between many species of *Vibrio*, leading to improved effectiveness and specificity of immune reactions (Tao Zhang *.et al.*, 2004; Netea MG, *.et al.*, 2019) , thus offering the possibility of specificity in primed responses. Repeated encounters with *Vibrio* bacteria lead to an increased and targeted immune response reactions (Tao Zhang *.et al.*, 2004; Netea MG, *.et al.*, 2019), which provides long-lasting protection against future *Vibrio* infections. Oysters have been seen to exhibit acquired immunity that can endure for a prolonged period, resulting in a decreased vulnerability to infections caused by *Vibrio* bacteria. The present study conducted an in vitro secondary challenge experiment, which revealed a significant decline in caring ability in both strains when sympatric-sympatric haemolymph was introduced. Indicative of a distinct immunological response targeting both strains.

Invertebrates have been subject to a long discussion regarding a possible immunological memory, and several reports have suggested that some forms of immune memory may exist in invertebrates, generally referred to as specific immune priming (Schmitt, P.,*et al.*, 2013). Most studies investigating immune priming focus on enhanced survival due to immune responses (Rowley, A.F

et al., 2007), which is observed in invertebrate immunity as a result of previous encounters with pathogens or their products, providing protection against reinfection known as "innate immune priming(Contreras Garduño, J., *et al.*, 2016). he knowledge of immune priming at the molecular and cellular levels for most observations in invertebrates is still limited. Oyster embryos and larvae have antibacterial and agglutination properties, with variations in antioxidant and hydrolytic enzymatic activities observed during ontogeny (Song, X *et al.*, 2016).Oysters contain antioxidant and hydrolytic enzymes, germline-encoded pathogen-associated pattern recognition receptors, and apoptosis and autophagy(Akira, S., *et al.*, 2006; Jemaà, M;*et al*,2014). The complex neuroendocrine-immune regulatory network uses neurotransmitters and hormones to regulate immune responses, with catecholamines playing a crucial role(Moreau, P., 2014; Chang, C.C *et al.*, 2015 ; Liu, Z., *et al.*, 2016).

However, oysters have been shown to have innate immune memory or priming, and such searches have led to several candidate mechanisms (Tao Zhang *et al.*, 2014 Song, X., *et al.*, 2016). Upon initial contact, oysters rapidly identify *Vibrio* bacteria by recognizing certain molecular patterns associated with these bacteria(Netea MG *et al.*, 2019). This initiates a series of immunological responses, which involve the activation of immune cells and the synthesis of antimicrobial substances(Tao Zhang *et al.*, 2014). The immune system of oysters acquires the ability to distinguish several species of *Vibrio*, resulting in enhanced efficiency and accuracy of immune responses(Netea MG *et al.*, 2019). Multiple exposures to *Vibrio* bacteria result in an augmented and specific immune response, which confers durable immunity against subsequent *Vibrio* infections. (Tao Zhang *et al.*, 2014 Song, X., *et al.*, 2016) The acquired immunity demonstrated by oysters may persist for an extended duration, hence reducing their susceptibility to infections triggered by *Vibrio* bacteria(Tao Zhang *et al.*, 2014 Song, X., *et al.*, 2016, Netea MG *et al.*, 2019).

Our research highlights the complex interactions between bacterial priming and oyster populations, providing insight into the complicated relationships among bacterial strains, priming sequences, and their effects on oyster health and bacterial growth. In this study, we have successfully shown that immune priming effectively induces immune responses in oysters against both sympatric and allopatric strains of *V. splendidus*. This immune priming approach aims to strengthen the oysters' particular memory and adaptive immunity. The acquired knowledge has the

potential to be applied in vaccination programs aimed at mitigating disease outbreaks within the aquaculture industry.

Outlook

The future prospects of this study include conducting more investigations into the underlying processes that drive immunological priming in oysters, as well as exploring potential strategies to use this phenomenon to augment their resistance to diseases. This phenomenon has the potential to facilitate the development of preventive measures against vibriosis in oyster populations, hence improving the total productivity of oyster harvesting.

conclusion

In summary, the three research endeavors discussed in this text collectively highlight the promising potential of natural resources and modern approaches in effectively addressing a range of challenges related to food safety, disease dynamics, and immune response. However, investigating hypotheses about the utilization of sea cucumber extracts, the interactions between *Vibrio* bacteria and oysters, and the processes of immune priming in oysters all indicate promising areas for further investigation and practical implementation.The examination of extracts from sea cucumber *Holothuria leucospilota*, the dynamics of *Vibrio* in *Crassostrea gigas*, and the immune responses of Pacific oysters *Magalana (Crassostrea) gigas* towards different strains of *Vibrio* collectively provide valuable insights into crucial facets of the health of marine invertebrates. These findings have significant ramifications for aquaculture and the sustainability of food production.

The potential antibacterial properties exhibited by sea cucumber extracts provide promising prospects for addressing seafood-related foodborne diseases and for the advancement of novel antimicrobial medicines. Although the current findings are promising, it is evident that further study is necessary in order to thoroughly explore the complete potential of these extracts and their active constituents.

These studies collectively enhance our comprehension of the dynamics of diseases affecting marine invertebrates and carry substantial consequences for the sustainability of aquaculture and

food production. Sea cucumber extracts have the potential to be utilized as a viable source of antimicrobial agents, thus providing a potential solution to the challenge of antibiotic resistance. The findings concerning the dynamics of *Vibrio* in oysters emphasize the necessity for customized disease management approaches in aquaculture in order to maintain the well-being and efficiency of oyster populations. Furthermore, the immune priming that was observed in Pacific oysters indicates the need for a nuanced strategy in disease prevention, demonstrating the significance of taking into account the particularities of host-pathogen interactions.

As global aquaculture plays an increasingly vital role in meeting the growing demand for seafood, understanding and managing diseases in marine invertebrates are paramount. The findings from these studies contribute valuable knowledge that can inform strategies for sustainable aquaculture practices, emphasizing the importance of exploring natural sources of antimicrobials, understanding host-pathogen interactions, and developing targeted disease management approaches. The implications of these studies extend beyond the immediate concerns of aquaculture and food sustainability to broader environmental considerations. Climate change, with its associated shifts in temperature and environmental conditions, could potentially influence the prevalence and impact of diseases in marine ecosystems. Future research in this field should consider the interconnected nature of climate change, marine invertebrate health, and the sustainability of aquaculture practices.

In conclusion, the exploration of sea cucumber extracts, *Vibrio* dynamics in oysters, and immune responses in Pacific oysters contributes to a holistic understanding of disease in marine invertebrates, offering valuable insights for sustainable aquaculture practices and raising important considerations for the broader context of climate change. Further research in these areas will be crucial for addressing the evolving challenges in marine health and ensuring the resilience of aquaculture systems.

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APPENDICES

Appendix chapter II

Antibacterial activity of the sea cucumber *Holothuria leucospilota* whole-body extract against methicillin-resistant and enterotoxin-producing *Staphylococcus aureus* strains

Statistical analysis

SPSS 19.0 (IBM, SPSS) was used for the analysis of variance of the raw data. Using Duncan's multiple range tests in ANOVA, we report all data as mean±SD. We also perform the least significant difference (LSD) tests to compare results among multiple groups.

Concentration (mg/ml). Hours (H).

The antimicrobial effect of chloroform extract of (N = 12) sea cucumbers *H. leucospilota* at different times (0,4,8,12,24) and concentrations. Optical density of extract was read by the ELISA reader at 650 nm.

All with MICs of about 50 mg/ml. values. The strains are ATCC 43300 (MRSA positive), ATCC 25923 (*sea* positive), and ATCC 14448 (*seb* positive), which were applied in the experiment without methanol or chloroform extract. 12 sea cucumber extracts were combined, and all experiments were run in triplicate

APPENDIX CHAPTER II

MRSA with sea cucumber chloroform extract

Concentration (mg/ml). Hours (H).

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	5	1.02668	0.205335	28.23	0.000
Linear	2	0.11115	0.055574	7.64	0.002
Time	1	0.08753	0.087535	12.03	0.001
concentration	1	0.01019	0.010187	6.40	0.045
Square	2	0.02718	0.013591	1.87	0.170
Time*Time	1	0.02458	0.024580	3.38	0.075
concentration *concentration	1	0.00260	0.002602	0.36	0.554
2-Way Interaction	1	0.30444	0.304442	41.85	0.000
Time*Concentration	1	0.30444	0.304442	41.85	0.000
Error	34	0.24732	0.007274		
Total	39	1.27400			

Concentrations of the chloroform extract showed minimum inhibitory towards a) the tested *S. aureus* strain MRSA with statistical results (max p<0.0 4; min F>1.87) and (R2=80.6%).

APPENDIX CHAPTER II

MRSA with Sea cucumber methanol extract

Concentration (mg/ml). Hours (H).

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	5	2.05297	0.410594	31.54	0.000
Linear	2	0.98035	0.490174	37.65	0.000
Time	1	0.66294	0.662936	50.93	0.002
concentration	1	0.17323	0.173233	13.31	0.001
Square	2	0.10353	0.051766	3.98	0.028
Time*Time	1	0.00097	0.000970	0.07	0.787
concentration *concentration					
0.008	7.88	0.102561	0.10256	1	
Way Interaction	1	0.12218	0.122178	9.39	0.004-2
Time*Concentration	1	0.12218	0.122178	9.39	0.004
Error	34	0.44261	0.013018		
Total	39	2.4955			

1. The antimicrobial effect of methanol extract of (N = 12) sea cucumbers *H. leucospilota* at different times (0,4,8,12,24) and concentrations. Optical density of extract was read by the ELISA reader at 650 nm. Concentrations of the methanol extract showed minimum inhibitory towards a) the tested *S. aureus* strain MRSA with statistical results (max $p<0.05$; min $F>13.3$),

SEA with sea cucumber chloroform extract

Concentration (mg/ml). Hours (H).

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	5	1.49271	0.298543	43.40	0.000
Linear	2	0.32070	0.160350	23.31	0.000
Time	1	0.17143	0.171434	24.92	0.000
concentration	1	0.09613	0.096125	13.97	0.001
Square	2	0.03385	0.016925	2.46	0.100
Time*Time	1	0.00050	0.000504	0.07	0.788
concentration *concentration	1	0.03335	0.033345	4.85	0.035
2-Way Interaction	1	0.39070	0.390699	56.80	0.000
Time*concentration	1	0.39070	0.390699	56.80	0.00
Error	34	0.23388	0.006879		
Total	39	1.72660			

The antimicrobial effect of chloroform extract of (N = 12) sea cucumbers *H. leucospilota* at different times (0,4,8,12,24) and concentrations. Optical density of extract was read by the ELISA reader at 650 nm.Concentrations of the chloroform extract showed minimum inhibitory towards a) the tested *S. aureus* strain SEASA with statistical results (max $p<0.05$; min $F>13.9$),

SEA with sea cucumber methanol extract

Concentration (mg/ml). Hours (H).

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	5	1.04897	0.209794	25.15	0.000
Linear	2	0.52165	0.260824	31.27	0.000
Time	1	0.32204	0.322045	38.61	0.001
concentration	1	0.11792	0.117919	14.14	0.003
Square	2	0.02334	0.011670	1.40	0.261
Time*Time	1	0.00674	0.006741	0.81	0.375
concentration *concentration	1	0.01660	0.016598	1.99	0.167
2-Way Interaction	1	0.07917	0.079167	9.49	0.004
Time* concentration	1	0.07917	0.079167	9.49	0.004
Error	34	0.28361	0.008341		
Total	39	1.33258			

1. The antimicrobial effect of methanol extract of (N = 12) sea cucumbers *H. leucospilota* at different times (0,4,8,12,24) and concentrations. Optical density of extract was read by the ELISA reader at 650 nm. Concentrations of the methanol extract showed minimum inhibitory towards a) the tested *S. aureus* strain SEASA with statistical results (max $p<0.05$; min $F>14.1$),

APPENDIX CHAPTER II

SEB with sea cucumber chloroform extract

Concentration (mg/ml). Hours (H).

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	5	0.200449	0.040090	26.88	0.000
Linear	2	0.072405	0.036202	24.27	0.000
Time	1	0.066722	0.066722	44.74	0.001
concentration	1	0.014006	0.014006	9.39	0.004
Square	2	0.000116	0.000058	0.04	0.962
Time*Time	1	0.000001	0.000001	0.00	0.983
concentration *concentration					
	0.783	0.08	0.000115	0.000115	1
Way Interaction	1	0.009732	0.009732	6.52	0.015-2
Time*Concentration	1	0.009732	0.009732	6.52	0.015
Error	34	0.050711	0.001491		
Total	39	0.251160			

The antimicrobial effect of chloroform extract of (N = 12) sea cucumbers *H. leucospilota* at different times (0,4,8,12,24) and concentrations. Optical density of extract was read by the ELISA reader at 650 nm.Concentrations of the chloroform extract showed minimum inhibitory towards a) the tested *S. aureus* strain SEBSA with statistical results (max $p<0.05$; min $F>9.3$).

SEB with Sea cucumber methanol extract

.

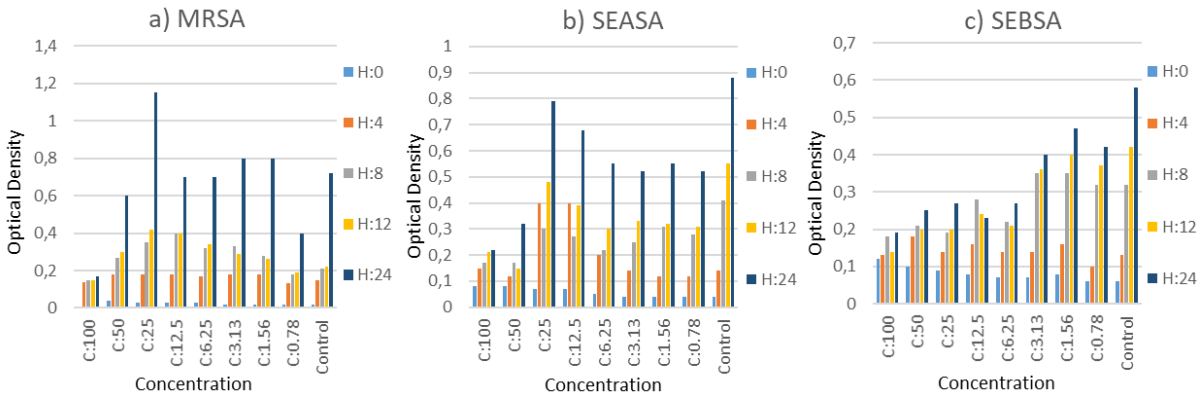
Concentration (mg/ml). Hours (H).

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	5	0.36611	0.073223	23.10	0.000
Linear	2	0.15938	0.079691	25.14	0.000
Time	1	0.07848	0.078485	24.75	0.002
concentration	1	0.05404	0.054036	17.04	0.001
Square	2	0.06169	0.030846	9.73	0.000
Time*Time	1	0.05169	0.051693	16.30	0.000
concentration *concentration	1	0.01000	0.010000	3.15	0.085
2-Way Interaction	1	0.03470	0.034696	10.94	0.002
Time*concentration	1	0.03470	0.034696	10.94	0.002
Error	34	0.10780	0.003170		
Total	39	0.47391			

The antimicrobial effect of methanol extract of (N = 12) sea cucumbers *H. leucospilota* at different times (0,4,8,12,24) and concentrations. Optical density of extract was read by the ELISA reader at 650 nm. Concentrations of the methanol extract showed minimum inhibitory towards a) the *S. aureus* strain SEBSA with statistical results (max $p<0.05$; min $F>17.0$).

APPENDIX CHAPTER II



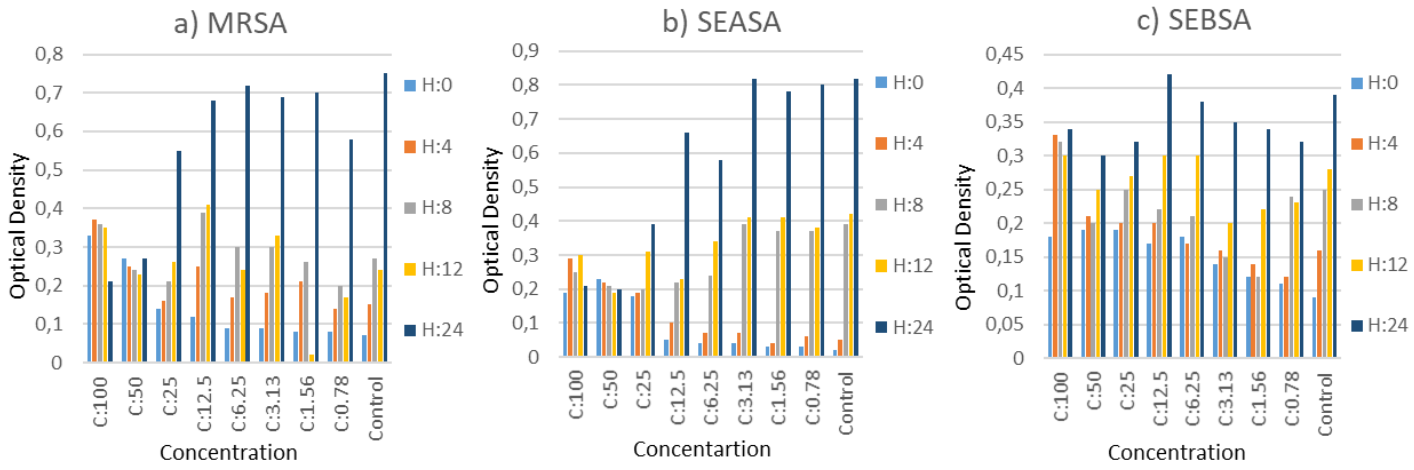
Concentration (mg/ml). Hours (H).

Figure 1. The antimicrobial effect of methanol extract of (N = 12) sea cucumbers *H. leucospilota* at different times (0,4,8,12,24 hrs) and concentrations. Optical density of extract was read by the ELISA reader at 650 nm. Concentrations of the methanol extract showed minimum inhibitory towards a) the tested *S. aureus* strain MRSA with statistical results (max $p<0.05$; min $F>13.3$), b) *S. aureus* strain SEASA with statistical results (max $p<0.05$; min $F>14.1$), and c) *S. aureus* strain SEBSA with statistical results (max $p<0.05$; min $F>17.0$),)(df total :39 ,df time –concentration:1) all with MICs of about 25 mg/ml. values. The strains are ATCC 43300 (MRSA positive), ATCC 25923 (*sea* positive), and ATCC 14448 (*seb* positive), which were applied in the experiment without methanol or chloroform extract. 12 sea cucumber extracts were combined, and all experiments were run in triplicate.

APPENDIX CHAPTER II

Strain	Time (hours)	Concentration (mg/ml)	Statistical Results (p-value, F-value)
MRSA	0 h	25	$p<0.05$; $F>13.3$
SEASA	4 h	25	$p<0.05$; $F>14.1$
SEBSA	8 h	25	$p<0.05$; $F>17.0$

Table 1. Illustrates the antimicrobial impact of the methanol extract from sea cucumbers *(Holothuria leucospilota*) at diverse concentrations and time points. The optical density of the extract was measured at 650 nm using an ELISA reader. The concentrations of the methanol extract demonstrated significant minimum inhibitory effects against *S. aureus* strains MRSA, SEASA, and SEBSA, each with distinct statistical outcomes (max $p<0.05$; min $F>13.3$, max $p<0.05$; min $F>14.1$, max $p<0.05$; min $F>17.0$, respectively))(df total :39 ,df time –concentration:1), all exhibiting minimum inhibitory concentrations (MICs) of approximately 25 mg/ml.



Concentration (mg/ml). Hours (H).

Figure 2. The antimicrobial effect of chloroform extract of (N = 12) sea cucumbers *H. leucospilota* at different times (0,4,8,12,24 hrs) and concentrations. Optical density of extract was read by the ELISA reader at 650 nm. Concentrations of the chloroform extract showed minimum inhibitory towards a) the tested *S. aureus* strain MRSA with statistical results (max $p<0.24$; min $F>1.4$) and ($R2=80.6\%$), b) *S. aureus* strain SEASA with statistical results (max $p<0.05$; min $F>13.9$), and c) *S. aureus* strain SEBSA with statistical results (max $p<0.05$; min $F>9.3$)(df total :39 ,df time –concentration:1) all with MICs of about 50 mg/ml. values. The strains are ATCC 43300 (MRSA positive), ATCC 25923 (*sea* positive), and ATCC 14448 (*seb* positive), which were applied in the experiment without methanol or chloroform extract. 12 sea cucumber extracts were combined, and all experiments were run in triplicate.

APPENDIX CHAPTER II

Strain	Time (hours)	Concentration (mg/ml)	Statistical Results (p-value, F-value)
MRSA	0	50	$p<0.24$; $F>1.4$
SEASA	4	50	$p<0.05$; $F>13.9$
SEBSA	8	50	$p<0.05$; $F>9.3$

Table 2. investigated the antimicrobial effects of the chloroform extract obtained from sea cucumbers (*Holothuria leucospilota*) at various concentrations (50 mg/ml) and time points (0, 4, 8, 12, 24 hours) against *Staphylococcus aureus* strains MRSA, SEASA, and SEBSA. (df total :39 ,df time –concentration:1) Optical density readings at 650 nm were recorded using an ELISA reader. The chloroform extract demonstrated minimum inhibitory concentrations (MICs) of approximately 50 mg/ml for all tested strains. Statistical analysis revealed significant inhibitory effects, with strain-specific results including maximum p-values below 0.24 and minimum F-values exceeding 1.4.

APPENDIX CHAPTER II

Table3. Disk diffusion result

Staphylococcus aureus	**Chloroform extract 40 µg/mL**	**Methanol extract 40 µg/mL**	**Vancomycin 30 µg/ml**	**Clindamycin 25 µg/ml**
MRSA	12 (mm)	14 (mm)	18 (mm)	16 (mm)
SEASA	9 (mm)	10 (mm)	19 (mm)	18 (mm)
SEBSA	12 (mm)	11 (mm)	18 (mm)	18 (mm)

Table 3. Inhibition zones for *S. aureus* strains MRSA, SEASA, and SEBSA under the effect of different extract of *H. leucospilota* with concentrations of 40 µg/mL and vancomycin, clindamycin by disk diffusion test. ≥17mm is sensitive zone for Vancomycin30 µg/ml and ≥ 14mm is sensitive zone for Clindamycin 25 µg/ml.

Table4. Antimicrobial activity of whole body of *H. leucospilota* methanol and chloroform extract

Staphylococcus aureus	***H. leucospilota* Extract**	**MIC**	**MBC**
MRSA	Methanol 40 µg/mL	>25 mg/ml	100 mg/ml
SEASA		>25 mg/ml	-
SEBSA		>25mg/ml	100 mg/ml
MRSA	Chloroform 40 µg/mL	>50 mg/ml	-
SEASA		>50 mg/ml	-
SEBSA		>50 mg/ml	100 mg/ml

Table4. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) from the whole body of *H. leucospilota* methanol and chloroform extract against *S. aureus* strains MRSA, SEASA, and SEBSA.

Appendix chapter III

Identification of *Vibrio kanaloae* in the oyster *Crassostrea gigas* by fluorescent in situ hybridization

APPENDIX CHAPTER III

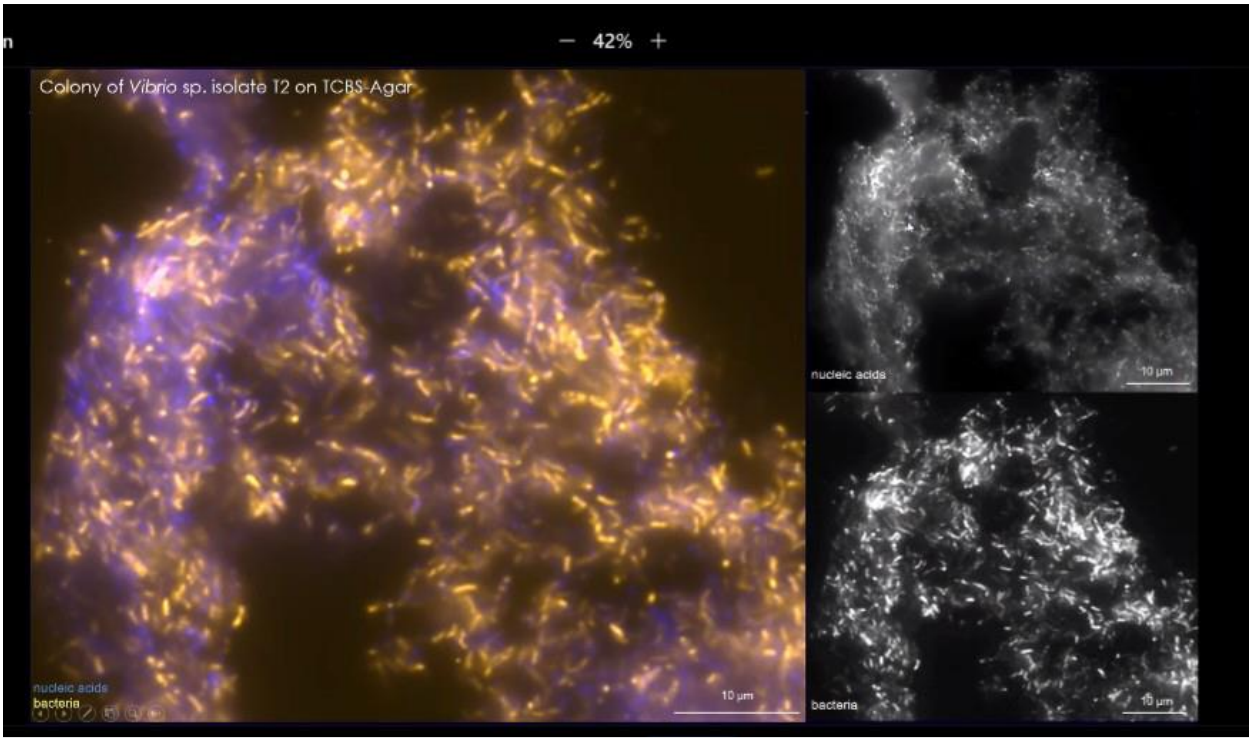


Figure 1: Fluorescence in situ hybridization (FISH) of illustrates *Vibrio kanakoea,* from bacteria growing on Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS)TCBS agar.

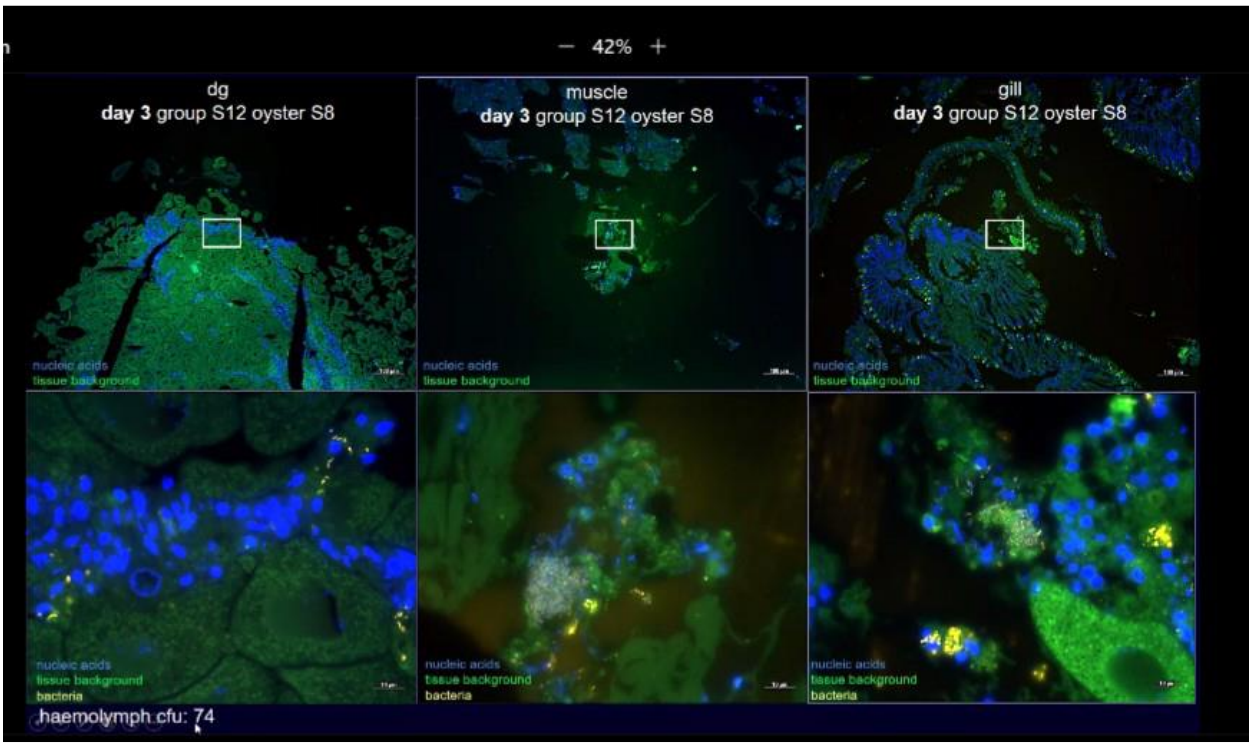


Figure 2 : Fluorescence in situ hybridization (FISH)Microscopic Analysis of *Vibrio* sympatric Strains S12 in Oyster Tissues on Day 3 Post-Infection. Employing Fluorescence in situ Hybridization (FISH), this figure captures the spatial distribution of *Vibrio* sympatric strains S12 within oyster tissues three days' post-infection. (A) The fluorescence signals, generated by the pan-bacterial FISH probe EUB338 (blue), vividly illustrate the infiltration of *Vibrio* sympatric strains S12 in the gill, digestive gland (df), and muscle tissues of the oyster. The background fluorescence in green provides context to the tissue. Zooming in on the inset (B), This visual representation offers an insight into the microbial colonization dynamics during the early stages of infection in oyster tissues.

	day 3 T2 digestive gland	day 3 T2 muscle	day 3 T2 gill
DAPI (nucleic acid stain) + morphotypes	positive	positive	positive
predominant morphotype	rods and spirochetes	long rods	multispecies
quantification	few	rare	few
pan-bacterial FISH probe	positive	negative	positive
location	at tissue borders	between muscle fibres	at tissue borders

Figure 3:

This figure illustrates the outcomes of the Fluorescence in situ Hybridization (FISH) technique, providing *Vibrio kanaloae* colonization in oyster tissues three days after infection. The results reveal differential patterns in DAPI staining and pan-bacterial FISH probe signals across distinct tissue types.

(A) Gill Tissue: DAPI staining showed a few positive signals, indicating the presence of microbial DNA. The pan-bacterial FISH probe demonstrated positive signals, suggesting active colonization by *Vibrio kanaloae*.

(B) Digestive Gland (df) Tissue: Similar to the gill, DAPI staining indicated a few positive signals, and the pan-bacterial FISH probe revealed positive signals, indicating *Vibrio kanaloae* colonization.

(C) Muscle Tissue: DAPI staining exhibited rare positive signals, suggesting a limited presence of microbial DNA. In contrast, the pan-bacterial FISH probe showed negative signals, indicating a lack of *Vibrio kanaloae* colonization in the muscle tissue.

APPENDIX CHAPTER IV

The co-evolutionary context of immune priming and cross-protection in the oyster *Magalana (Crassostrea) gigas*

APPENDIX CHAPTER IV

Primary Challenge

72 Pacific Oyster *Magalana (Crassostrea) gigas*

Divided to three groups (W, T and Control)

In vivo examination

Oysters exposed with 10^4 cell of *V. splendidus* T (Tx5.1, allopatric) and W (O7w-July, sympatric) strains

24 W 24 T 24 Control

Obtaining the oysters serums day 1,3,5 post infection

In Vitro examination

Examination in microplate reader ,Primary oysters serums day 1,3,5 post infection incubated 24 h with 10^5 cell of *vibro splendidus* sympatric, allopatric strains.

W T Control

Secondary challenge

In vivo examination

Oysters exposed with 10^7 cell of *V. splendidus* T (Tx5.1, allopatric) and W (O7w-July, sympatric) strains

8 WW 8 WT 8 WC 8 TT 8 TW 8 TC

8 CC 8 CT 8 CW

Obtaining the Oysters serums day 1,3,5 post infection

In Vitro examination

Examination in microplate reader , oysters serums day 1,3,5 post infection incubated 24 h with 10^5 cell of *vibro splendidus* sympatric, allopatric strains.

WW WT WC TT T W TC

CC CT CW

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Figure 1:
The depicted figure delineates primary and secondary challenges within a sequence of both in vivo and in vitro experiments conducted on Pacific oysters *Magalana (Crassostrea) gigas*. In the primary in vivo phase, oysters were subjected to infection with *Vibrio splendidus* strains sympatric or W (O7w_July) and allopatric or T(Tx5.1). During the primary in vitro experiments, haemolymph obtained from individually exposed oysters served as a pivotal component for evaluating the immune response. The haemolymph serum underwent meticulous filter-sterilization and subsequent incubation with *V. splendidus* strains with a microplate reader, followed by the measurement of resulting growth curves over 24 hours.
Subsequently, in the secondary challenge, oysters involved the division of primary challenge groups into three subsets. These subsets were subjected to infection with either sympatric or allopatric *Vibrio* strains within the treatment groups, while the control group received a nutrient medium. Haemolymph was systematically collected once more to ascertain bacterial load.The secondary in vitro challenge, designed to study cross-protection, is carried on the experimental setup of the primary experiment. Haemolymph was categorized into nine distinct groups and strategically placed within a 96-well plate. Bacterial strains were introduced, and ensuing growth curves were meticulously monitored

represents a Pacific oyster *Magalana* (*Crassostrea gigas*).

represents a microplate icon for in vitro challenges.

represents icon for oyster's serum.

APPENDIX CHAPTER IV

Supplementary material:

Table 1. AIC model comparison for Single exposure in vitro inhibition

Model selection based on
AICc:

	K	AICc	Delta_AICc	AICcWt	Cum.Wt	LL
medium*day*strain	19	143.24	0	0.84	0.84	-41.44
medium	4	147.4	4.16	0.11	0.95	-69.29
medium+day+strain	7	148.97	5.73	0.05	0.99	-66.27
medium*strain	7	153.31	10.07	0.01	1	-68.44
day*strain	7	163.1	19.86	0	1	-73.33

Table S2. Analysis of Variance Table

Response: knorm

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
medium	2	11.064	5.532	13.5719	4.05E-05	***
day	2	4.3089	2.1544	5.2856	0.009713	**
strain	1	0.0539	0.0539	**0.1324**	**0.718135**	
medium:day	4	15.3568	3.8392	9.4188	2.57E-05	***
medium:strain	2	1.2251	0.6125	1.5027	0.236149	
day:strain	2	0.0576	0.0288	0.0707	0.931912	
medium:day:strain	4	5.4834	1.3708	**3.3631**	**0.019437**	*
Residuals	36	14.6739	0.4076			

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Table S3. Post-hoc tests.

Tukey HSD every comparison

Tukey multiple
comparisons of means
95% family-wise
confidence level

Fit: aov(formula = knorm ~ day * medium *
strain, data = curves.statsf)

$`day:medium:strai
n`

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1d	1h	1b	2d	2h	2b	diff	lwr	upr	p adj
D3	HC	T	D1	HC	T	2.22432095	0.2693155	4.1793263	0.0132619
D5	HC	T	D1	HC	T	0.7921152	-1.1628901	2.7471205	0.983089
D5	HC	T	D3	HC	T	-1.4322057	-3.3872111	0.5227996	0.386252
D1	HC	W	D1	HC	T	-0.1287296	-2.0837349	1.8262757	1
D1	HC	W	D3	HC	T	-2.3530505	-4.3080559	-0.3980452	0.0067553
D1	HC	W	D5	HC	T	-0.9208448	-2.8758502	1.0341605	0.937772
D3	HC	W	D1	HC	T	1.76877487	-0.1862305	3.7237802	0.114295
D3	HC	W	D3	HC	T	-0.4555461	-2.4105514	1.4994592	0.999977
D3	HC	W	D5	HC	T	0.97665964	-0.9783457	2.9316649	0.903502
D5	HC	W	D1	HC	T	1.22924152	-0.7257638	3.1842468	0.639867
D5	HC	W	D3	HC	T	-0.9950794	-2.9500848	0.9599259	0.890026
D5	HC	W	D5	HC	T	0.43712628	-1.5178791	2.3921316	0.999987
D3	HC	W	D1	HC	W	-1.89750444	-3.8525097	0.0575009	0.065143
D5	HC	W	D1	HC	W	-1.35797108	-3.3129764	0.5970343	0.4752501
D5	HC	W	D3	HC	W	-0.5395334	-2.4945387	1.415472	0.9997777
D1	HC	W	D1	HT	T	-2.8216463	-4.7766517	-0.866641	0.0005078
D1	HC	W	D3	HT	T	-1.7896704	-3.7446757	0.1653349	0.104646
D1	HC	W	D5	HT	T	-1.8887094	-3.8437148	0.0662959	0.067786
D3	HC	W	D1	HT	T	-0.9241419	-2.8791473	1.0308634	0.936018
D3	HC	W	D3	HT	T	0.10783408	-1.8471713	2.0628394	1
D3	HC	W	D5	HT	T	0.00879504	-1.9462103	1.9638003	1
D5	HC	W	D1	HT	T	-1.4636753	-3.4186806	0.4913300	0.351131
D5	HC	W	D3	HT	T	-0.4316993	-2.3867046	1.5233060	0.999989
D5	HC	W	D5	HT	T	-0.5307383	-2.4857437	1.4242670	0.999820
D1	HC	W	D1	HW	T	-2.1386335	-4.0936389	-0.1836282	0.020511
D1	HC	W	D3	HW	T	-2.0482188	-4.0032242	-0.0932135	0.032066

D1	HC	W	D5	HW	T	-1.3703757	-3.3253811	-0.5846296	0.459875
D3	HC	W	D1	HW	T	-0.2411291	-2.1961345	-1.7138762	1
D3	HC	W	D3	HW	T	-0.1507144	-2.1057198	-1.8042909	1
D3	HC	W	D5	HW	T	0.5271286	-1.4278767	-2.4821340	0.999835
D5	HC	W	D1	HW	T	-0.7806625	-2.7356678	-1.1743429	0.985285
D5	HC	W	D3	HW	T	-0.6902478	-2.6452531	-1.2647575	0.995833
D5	HC	W	D5	HW	T	-0.0124047	-1.96741	-1.9426006	1
D1	HT	T	D1	HC	T	2.6929167	0.7379114	4.6479221	0.001049
D1	HT	T	D3	HC	T	0.4685958	-1.4864095	-2.4236011	0.999966
D1	HT	T	D5	HC	T	1.9008015	-0.0542038	-3.8558069	0.064175
D3	HT	T	D1	HC	T	1.6609407	-0.2940646	-3.6159461	0.176417
D3	HT	T	D3	HC	T	-0.5633802	-2.5183855	-1.3916252	0.999615
D3	HT	T	D5	HC	T	0.8688255	-1.0861798	-2.8238309	0.961226
D5	HT	T	D1	HC	T	1.7599798	-0.1950255	-3.7149851	0.118573
D5	HT	T	D3	HC	T	-0.4643411	-2.4193465	-1.4906642	0.99997
D5	HT	T	D5	HC	T	0.9678646	-0.9871408	-2.9228699	0.909556
D3	HT	T	D1	HT	T	-1.031976	-2.9869813	-0.9230293	0.859816
D5	HT	T	D1	HT	T	-0.9329369	-2.8879423	-1.0220684	0.931175
D5	HT	T	D3	HT	T	0.0990390	-1.8559663	-2.0540444	1
D1	HT	W	D1	HC	T	1.3725814	-0.582424	3.3275867	0.457159
D1	HT	W	D3	HC	T	-0.8517395	-2.8067449	-1.1032658	0.967299
D1	HT	W	D5	HC	T	0.5804661	-1.3745392	-2.5354715	0.999442
D3	HT	W	D1	HC	T	2.3697250	0.4147197	4.3247304	0.006180
D3	HT	W	D3	HC	T	0.1454041	-1.8096013	-2.1004094	1
D3	HT	W	D5	HC	T	1.5776098	-0.3773955	-3.5326151	0.240244
D5	HT	W	D1	HC	T	1.6618734	-0.2931319	-3.6168788	0.175784
D5	HT	W	D3	HC	T	-0.5624475	-2.5174529	-1.3925578	0.999623

D5	HT	W	D5	HC	T	0.8697582	-1.0852471	2.8247635	0.960872
D1	HT	W	D1	HC	W	1.5013109	-0.4536944	3.4563163	0.311564
D1	HT	W	D3	HC	W	-0.3961935	2.3511988	-1.5588118	0.999996
D1	HT	W	D5	HC	W	0.1433398	-1.8116655	2.0983452	1
D3	HT	W	D1	HC	W	2.4984546	0.5434492	4.4534599	0.003082
D3	HT	W	D3	HC	W	0.6009501	-1.3540552	2.5559555	0.999148
D3	HT	W	D5	HC	W	1.1404835	-0.8145218	3.0954888	0.748553
D5	HT	W	D1	HC	W	1.7906030	-0.1644023	3.7456083	0.104232
D5	HT	W	D3	HC	W	-0.1069014	2.0619068	-1.8481039	1
D5	HT	W	D5	HC	W	0.4326319	-1.5223734	2.3876372	0.999988
D1	HT	W	D1	HT	T	-1.3203354	3.2753407	-0.6346699	0.522779
D1	HT	W	D3	HT	T	-0.2883594	2.2433648	-1.6666459	1
D1	HT	W	D5	HT	T	-0.3873984	2.3424038	-1.5676069	0.999997
D3	HT	W	D1	HT	T	-0.3231917	2.2781971	-1.6318136	0.999999
D3	HT	W	D3	HT	T	0.7087842	-1.2462211	2.6637896	0.994464
D3	HT	W	D5	HT	T	0.6097452	-1.3452601	2.5647505	0.998986
D5	HT	W	D1	HT	T	-1.0310433	2.9860487	-0.9239620	0.860631
D5	HT	W	D3	HT	T	0.0009326	-1.9540727	1.9559380	1
D5	HT	W	D5	HT	T	-0.0981064	2.0531117	-1.8568989	1
D3	HT	W	D1	HT	W	0.9971436	-0.9578617	2.9521490	0.888449
D5	HT	W	D1	HT	W	0.2892920	-1.6657133	2.2442974	1
D5	HT	W	D3	HT	W	-0.7078516	-2.662857	-1.2471537	0.994540
D1	HT	W	D1	HW	T	-0.6373226	2.5923279	-1.3176827	0.998296
D1	HT	W	D3	HW	T	-0.5469079	2.5019132	-1.4080974	0.999735
D1	HT	W	D5	HW	T	0.1309352	-1.8240701	2.0859405	1
D3	HT	W	D1	HW	T	0.3598210	-1.5951843	2.3148264	0.999999
D3	HT	W	D3	HW	T	0.4502357	-1.5047696	2.4052411	0.999980

D3	HT	W	D5	HW	T	1.1280788	-0.8269265	3.0830842	0.762730
D5	HT	W	D1	HW	T	-0.3480305	-2.3030359	-1.6069748	0.999999
D5	HT	W	D3	HW	T	-0.2576158	-2.2126212	-1.6973895	1
D5	HT	W	D5	HW	T	0.4202272	-1.5347781	2.3752326	0.999992
D1	HW	T	D1	HC	T	2.0099039	0.0548986	3.9649093	0.038573
D1	HW	T	D3	HC	T	-0.214417	-2.1694223	-1.7405883	1
D1	HW	T	D5	HC	T	1.2177887	-0.7372166	3.1727940	0.654414
D3	HW	T	D1	HC	T	1.9194892	-0.0355161	3.8744946	0.058929
D3	HW	T	D3	HC	T	-0.3048317	-2.259837	-1.6501736	0.999999
D3	HW	T	D5	HC	T	1.1273740	-0.8276313	3.0823794	0.763526
D5	HW	T	D1	HC	T	1.2416461	-0.7133592	3.1966515	0.624011
D5	HW	T	D3	HC	T	-0.9826748	-2.9376801	-0.9723305	0.899220
D5	HW	T	D5	HC	T	0.4495309	-1.5054744	2.4045363	0.999980
D1	HW	T	D1	HT	T	-0.6830128	-2.6380182	-1.2719925	0.996285
D1	HW	T	D3	HT	T	0.3489631	-1.6060422	2.3039685	0.999999
D1	HW	T	D5	HT	T	0.2499241	-1.7050812	2.2049294	1
D3	HW	T	D1	HT	T	-0.7734275	-2.7284329	-1.1815778	0.986553
D3	HW	T	D3	HT	T	0.2585484	-1.6964569	2.2135538	1
D3	HW	T	D5	HT	T	0.1595094	-1.7954959	2.1145148	1
D5	HW	T	D1	HT	T	-1.4512706	-3.406276	-0.5037347	0.364764
D5	HW	T	D3	HT	T	-0.4192946	-2.3743	-1.5357107	0.999992
D5	HW	T	D5	HT	T	-0.5183337	-2.473339	1.4366717	0.999867
D3	HW	T	D1	HW	T	-0.0904147	-2.04542	-1.8645906	1
D5	HW	T	D1	HW	T	-0.7682578	-2.7232631	-1.1867475	0.987405
D5	HW	T	D3	HW	T	-0.6778431	-2.6328485	-1.2771622	0.996582
D1	HW	W	D1	HC	T	3.3744965	1.4194911	5.3295018	0.000021
D1	HW	W	D3	HC	T	1.1501755	-0.8048298	3.1051809	0.737263

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D1	HW	W	D5	HC	T	2.5823812 7	0.6273759 1	4.5373866 2	0.001942 4
D3	HW	W	D1	HC	T	2.0282259 8	0.0732206 2	3.9832313 3	0.035325
D3	HW	W	D3	HC	T	-0.196095	2.1511003	-1.7589103 8	1
D3	HW	W	D5	HC	T	1.2361107 4	0.7188946	-3.1911161	0.631098
D5	HW	W	D1	HC	T	1.1940565 9	0.7609488	-3.1490619 4	0.684175 3
D5	HW	W	D3	HC	T	-1.0302644	-2.9852697	-0.924741	0.861310 9
D5	HW	W	D5	HC	T	0.4019413 6	-1.553064	2.3569467 1	0.999996 1
D1	HW	W	D1	HC	W	3.5032260 7	1.5482207 1	5.4582314 2	0.000010 2
D1	HW	W	D3	HC	W	1.6057216 3	0.3492837	-3.5607269 8	0.217079 4
D1	HW	W	D5	HC	W	2.1452549 9	0.1902496 3	4.1002603 4	0.019839 7
D3	HW	W	D1	HC	W	2.1569555 4	0.2019501 9	4.1119609	0.018703 2
D3	HW	W	D3	HC	W	-0.2594511	-1.6955543	-2.2144564 6	1
D3	HW	W	D5	HC	W	0.7989844 6	-1.1560209	-2.7539898 1	0.981654 3
D5	HW	W	D1	HC	W	1.3227861 6	0.6322192	-3.2777915 1	0.519651 5
D5	HW	W	D3	HC	W	-0.5747183	-2.5297236	-1.3802870 7	0.999506 5
D5	HW	W	D5	HC	W	-0.0351849	-1.9901903	-1.9198204 3	1
D1	HW	W	D1	HT	T	0.6815797 2	-1.2734256	-2.6365850 8	0.996369 8
D1	HW	W	D3	HT	T	1.7135557 1	-0.2414497	-3.6685610 6	0.143398 6
D1	HW	W	D5	HT	T	1.6145166 7	-0.3404887	-3.5695220 2	0.210176 5
D3	HW	W	D1	HT	T	-0.6646908	-2.6196962	-1.2903145 5	0.997250 8
D3	HW	W	D3	HT	T	0.3672851 8	-1.5877202	-2.3222905 4	0.999999
D3	HW	W	D5	HT	T	0.2682461 4	-1.6867592	-2.2232514 9	1
D5	HW	W	D1	HT	T	-1.4988602	-3.4538655	-0.4561451 7	0.314056 2
D5	HW	W	D3	HT	T	-0.4668842	-2.4218896	-1.4881211 5	0.999967 6
D5	HW	W	D5	HT	T	-0.5659232	-2.5209286	-1.3890821 1	0.999592 8
D1	HW	W	D1	HT	W	2.0019151	0.0469097 4	3.9569204 5	0.040073 8
D1	HW	W	D3	HT	W	1.0047714 5	-0.9502339	-2.9597768	0.882503 5

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D1		HW	W	D5	HT	W	1.7126230 6	-0.2423823	3.6676284 1	0.143937 5
D3		HW	W	D1	HT	W	0.6556445 7	-1.2993608	2.6106499 3	0.997643 7
D3		HW	W	D3	HT	W	-0.3414991	-2.2965044	1.6135062 8	0.999999 7
D3		HW	W	D5	HT	W	0.3663525 3	-1.5886528	2.3213578 8	0.999999
D5		HW	W	D1	HT	W	-0.1785248	-2.1335302	1.7764805 4	1
D5		HW	W	D3	HT	W	-1.1756685	-3.1306738	0.7793368 9	0.706776 4
D5		HW	W	D5	HT	W	-0.4678169	-2.4228222	1.4871885	0.999966 7
D1		HW	W	D1	HW	T	1.3645925 3	-0.5904128	3.3195978 9	0.467022 1
D1		HW	W	D3	HW	T	1.4550072 2	-0.4999981	3.4100125 8	0.360628 1
D1		HW	W	D5	HW	T	2.1328503 2	0.1778449 6	4.0878556 7	0.021114 9
D3		HW	W	D1	HW	T	0.0183220 1	-1.9366834	1.9733273 6	1
D3		HW	W	D3	HW	T	-0.1087367	-1.8462687	2.0637420 5	1
D3		HW	W	D5	HW	T	0.7865797 9	-1.1684256	2.7415851 5	0.984180 4
D5		HW	W	D1	HW	T	-0.8158474	-2.7708527	1.1391579 8	0.977733 5
D5		HW	W	D3	HW	T	-0.7254327	-2.680438	1.2295726 7	0.992938 1
D5		HW	W	D5	HW	T	-0.0475896	-2.002595	1.9074157 6	1
D3		HW	W	D1	HW	W	-1.3462705	-3.3012759	0.6087348 3	0.489898
D5		HW	W	D1	HW	W	-2.1804399	-4.1354453	-0.2254346	0.016603 8
D5		HW	W	D3	HW	W	-0.8341694	-2.7891747	1.1208359 7	0.972782 4

Single Exposure in vivo CFU

Table S4. Analysis of deviance table, negative binomial GLM

Analysis of Deviance Table (Type II tests)

Response	CFU			
LR	Chisq	Df	Pr(>Chisq)	
Day	13.178	2	0.001375	**
Strain	38.331	2	4.75E-09	***
Day*Strain	12	4	0.017349	*

Table S5. Posthoc comparison of treatments with emmeans

contrast	estimate	SE	df	z.ratio	p.value
C - T	-1.6	0.54	Inf	-2.962	0.0086
C - W	-3.14	0.531	Inf	-5.913	<.0001
T - W	-1.54	0.398	Inf	-3.871	0.0003

Table S5: Pairwise comparisons of strain within day with emmeans

$contrasts

Strain = C

contrast	estimate	SE	df	z.ratio	p.value
Day1 - Day3	2.241	0.807	Inf	2.777	0.0152
Day1 - Day5	3.85	1.205	Inf	3.196	0.004
Day3 - Day5	1.609	1.277	Inf	1.261	0.4175

Strain = T

contrast	estimate	SE	df	z.ratio	p.value
Day1 - Day3	1.099	0.716	Inf	1.534	0.2751
Day1 - Day5	0.26	0.691	Inf	0.377	0.9248
Day3 - Day5	-0.838	0.721	Inf	-1.163	0.4752

Strain = W

contrast	estimate	SE	df	z.ratio	p.value
Day1 - Day3	0.318	0.663	Inf	0.481	0.8805
Day1 - Day5	1.406	0.671	Inf	2.097	0.0905
Day3 - Day5	1.088	0.672	Inf	1.619	0.2374

Table S6: Negative binomial GLM primary and secondary exposure

Analysis of Deviance Table (Type II tests)

Response	CFU			
	LR Chisq	Df	Pr(>Chisq)	
Primary_Strain	6.0086	2	0.049574	*
Secondary_Strain	8.3643	2	0.015266	*
Day	1.4118	2	0.493675	
Primary_Strain*Secondary_Strain	11.1957	4	0.024451	*
Primary_Strain*Day	1.469	4	0.832115	
Secondary_Strain*Day	3.408	4	0.492002	
Primary_Strain*Secondary_Strain*Day	24.8251	8	0.001664	**

Table S7. Multiple comparison tests for the second in vivo experiment

$contrasts

Primary_Strain = C

contrast	estimate	SE	df	z.ratio	p.value
C - T	-0.944	0.625	Inf	-1.51	0.286
C - W	0.954	0.634	Inf	1.505	0.2886
T - W	1.898	0.632	Inf	3.005	0.0075

Primary_Strain = T

contrast	estimate	SE	df	z.ratio	p.value
C - T	-2.159	0.637	Inf	-3.389	0.002
C - W	-0.757	0.642	Inf	-1.178	0.4663
T - W	1.402	0.624	Inf	2.245	0.0638

Primary_Strain = W

contrast	estimate	SE	df	z.ratio	p.value
C - T	-0.821	0.65	Inf	-1.263	0.4163
C - W	-1.948	0.66	Inf	-2.951	0.0089
T - W	-1.127	0.643	Inf	-1.752	0.186

Table S8. AIC model comparison for the second in vitro experiment

Model selection based on
AICc

	K	AICc	Delta_AICc	AICcWt	Cum.Wt	Res.LL
primary*secondary	11	335.86	0	1	1	-156.05
primary*secondary*strain	20	362.15	26.29	0	1	-158.09
secondary	5	374.92	39.06	0	1	-182.27
primary+secondary+strain	8	384.52	48.66	0	1	-183.79
primary	5	432.19	96.33	0	1	-210.9

Table S9. Multiple comparisons for the second in vitro experiment

$contrasts
secondary = SC

contrast	estimate	SE	df	t.ratio	p.value
PT - PC	0.2272	0.2	151	1.136	0.4935
PT - PW	0.2092	0.2	151	1.046	0.5494
PC - PW	-0.0181	0.2	151	-0.09	0.9955

secondary = ST

contrast	estimate	SE	df	t.ratio	p.value
PT - PC	0.8165	0.2	151	4.082	0.0002
PT - PW	-0.3436	0.2	151	-1.718	0.2019
PC - PW	-1.1602	0.2	151	-5.8	<.0001

secondary = SW

contrast	estimate	SE	df	t.ratio	p.value
PT - PC	-0.3446	0.2	151	-1.723	0.2
PT - PW	0.8172	0.2	151	4.085	0.0002
PC - PW	1.1619	0.2	151	5.808	<.0001

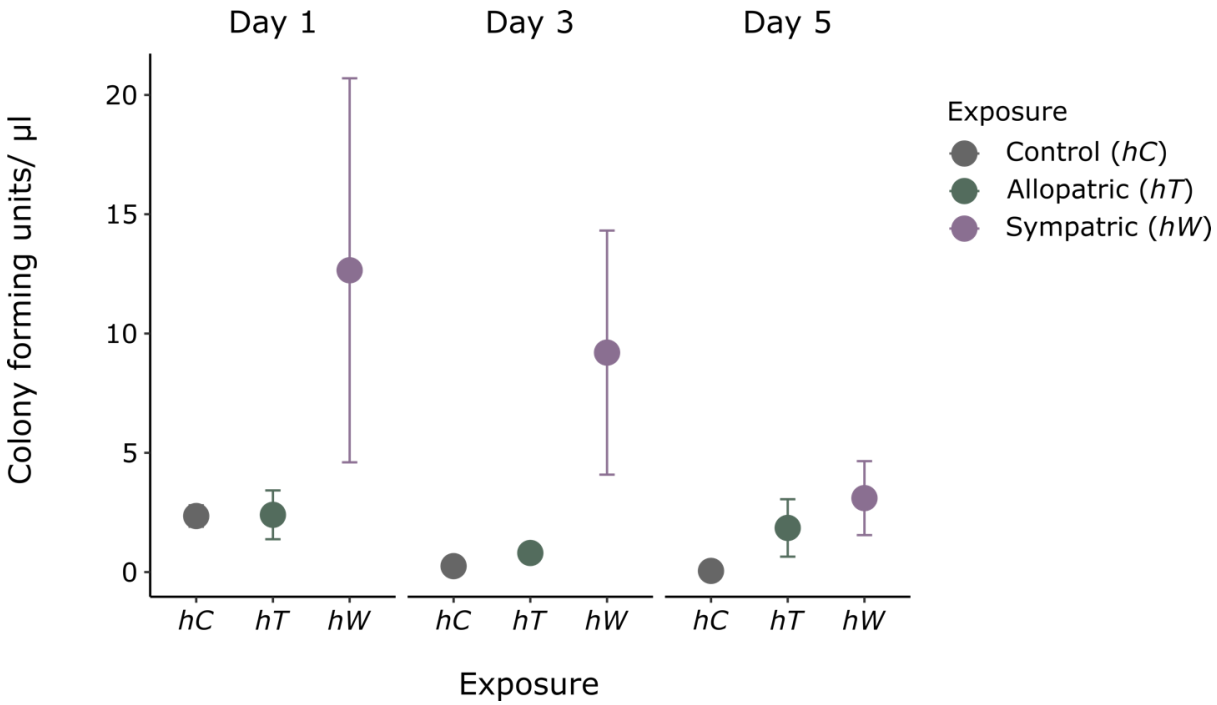

Figure 2. *In vivo* experiment priming in the two infection groups W *(* infected by the sympatric *Vibrio* O7w_July) and group T (infected by the allopatric *Vibrio* Tx5.1 as well as the control group of oysters on day 1,3 and 5 post infection. Challenge with sympatric *Vibrio vW* resulted in higher bacterial loads than allopatric *Vibrio* or control inoculation and injection with allopatric bacteria resulted in higher bacteria loads than the control treatment only on day 5.

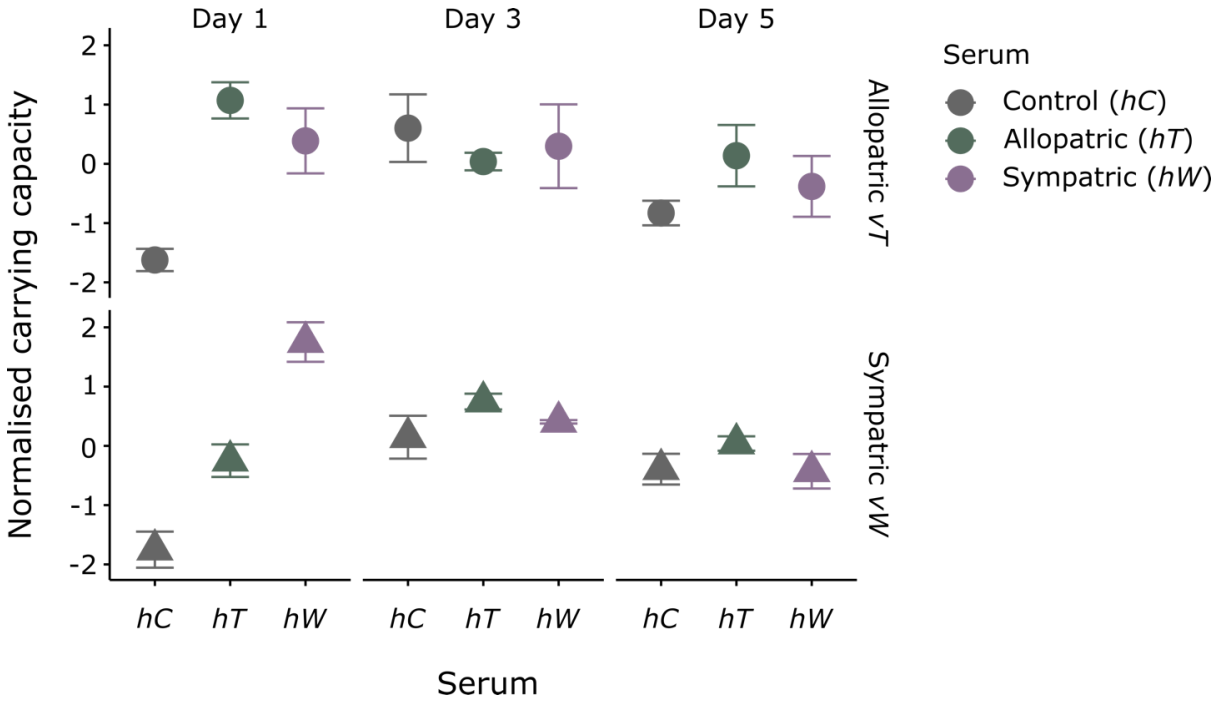

Figure 3: Carrying capacities of hemolymph serum primed with different exposures (hW, hC and hT) for *Vibrio* strains vT and vW after 1,3 and 5 days post infection . Primary exposure with either strain increased carrying capacity, but was strongest for matching combination of Vibrio strain and hemolymph on day 1 (vW – hW, vT-hT). Highest carrying capacity was reached for the vW-hW combination of day 1, but decreased significantly over time, whereas low carrying capacities were only found in hC on day one, but also increased significantly with allopatric bacteria (Tukey test<0.05), and showed a trend with sympatric bacteria (Tukey test p=0.065) throughout the course of the experiment

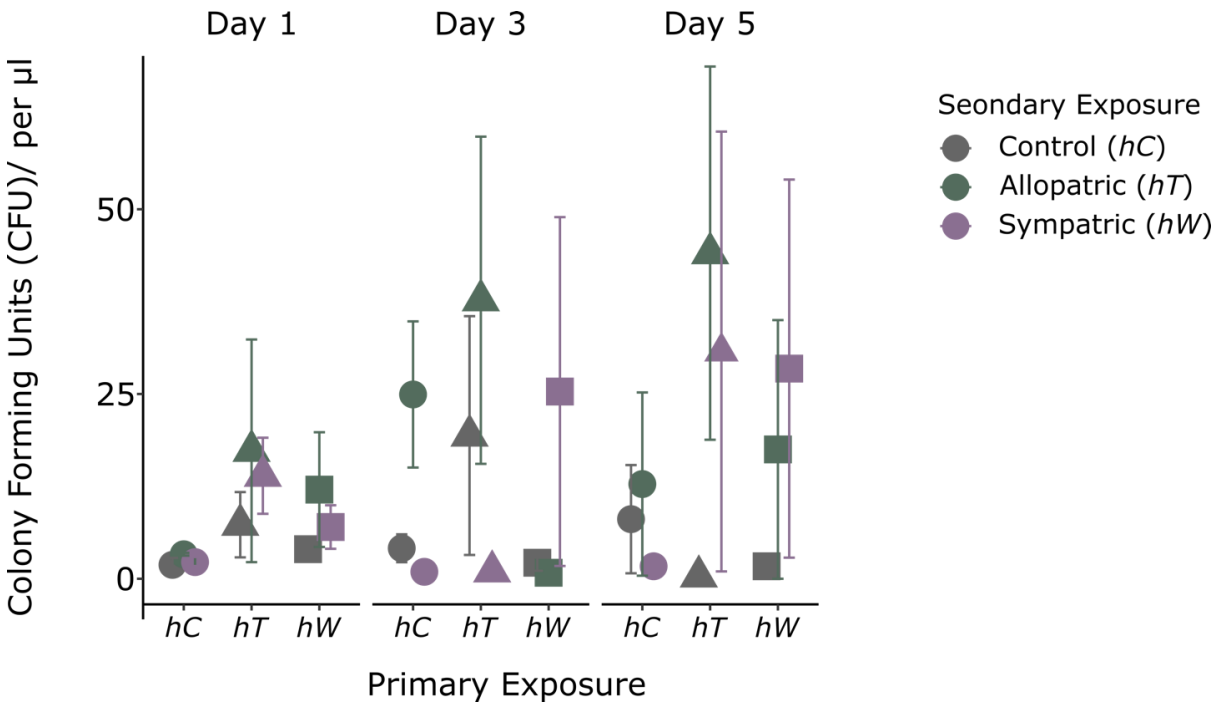

Figure 4. Bacterial load after the secondary challenge of oysters primed with sympatric (vW) and allopatric (vT) *V. splendidus.* A significant interaction between the primary and secondary exposures in the *in vivo* experiment (Negative binomial GLM: Chisq = 11.2, d.f. = 4, p = 0.024) indicates that priming influences the response to subsequent exposures. Oysters primed with sympatric or allopatric bacteria showed significantly higher bacterial loads when exposed twice, suggesting priming increases in vivo growth rather than inhibiting it.

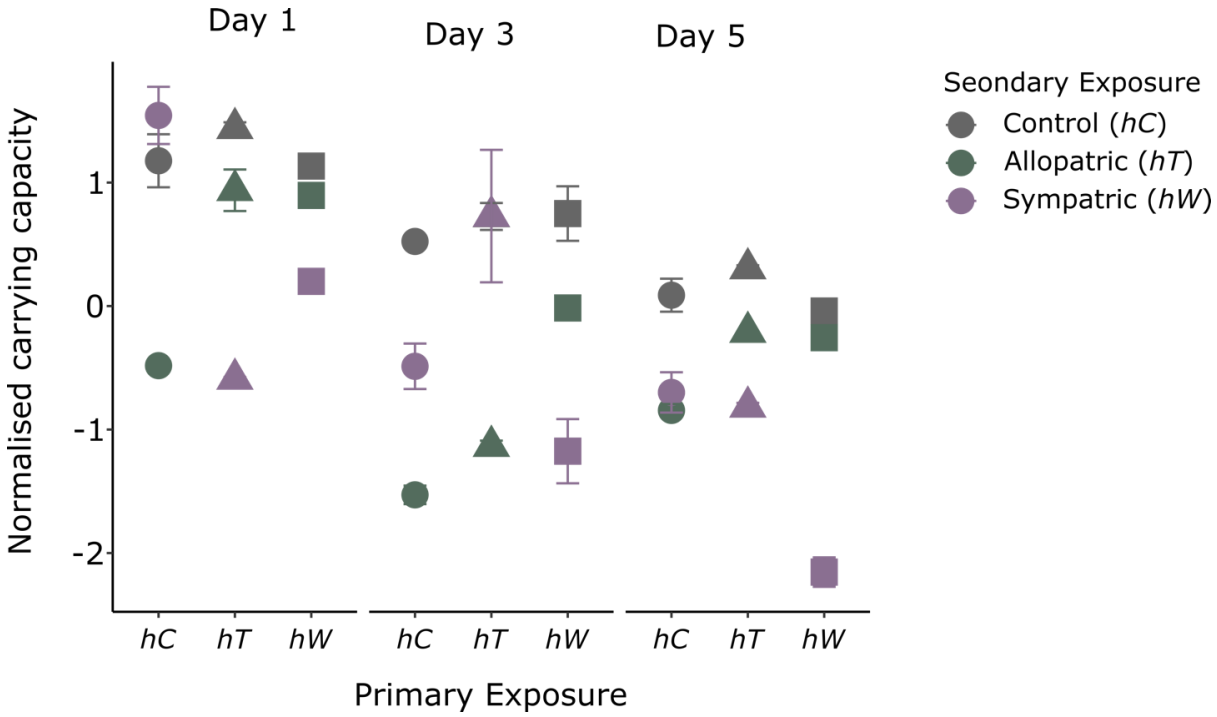

Figure 5. Carrying capacities of hemolymph serum after priming and secondary exposure with *Vibrio* strains vT and vW after 1,3 and 5 days post infection data from growth with vT and vW were pooled for simplification as the strain being grown hat no significant impact on the outcome and was exclused in the best fitting model. **T**he interaction between primary and secondary infection was significant (Nested linear model: Chisq = 67.4, d.f.= 4, $p < 0.0001$). Within the control secondary treatment there were no significant differences among primary treatments. Within the allopatric secondary treatment, both primary treatment with allopatric and sympatric bacteria resulted in significantly higher carrying capacities than the control (Tukey multiple comparison tests $p<0.05$) but did not differ from one another.

ACKNOWLEDGEMENTS

I extend my deepest gratitude to Professor Dr. Jens Rolff for his exceptional contribution to the successful completion of this thesis. His gracious invitation to join his research group has played a pivotal role in the project's accomplishment. Professor Rolff has been a source of inspiration and guidance, entrusting me with significant responsibilities and fostering an intellectually stimulating environment throughout my academic journey. His mentorship has been truly invaluable, providing me with profound insights and unwavering motivation. This thesis stands as a testament to the profound impact of his guidance and support.

I would like to express my sincere gratitude to Dr. Mathias K. Wegner for his exceptional guidance and support during my experiment dissertation at Alfred Wegener Institute (AWI). Dr. Wegner's expertise and insightful mentoring have been instrumental in the success of this research endeavor. I am thankful for his dedication to fostering a collaborative and enriching research environment, providing valuable insights, and encouraging innovative thinking. This dissertation represents the culmination of his mentorship, and I am truly appreciative of the knowledge and skills gained under his supervision. The experience has been both rewarding and enlightening, shaping my academic and research journey in significant ways.

With remembrance and commemoration, to the late Dr. Ms. Azar Andami (1926-1984) of Tehran University of Medical Sciences, Institute Pasteur. Her groundbreaking discovery of the *Vibrio* Eltor vaccination in 1966 stands as a beacon of inspiration. This monumental achievement has left an indelible mark on public health, saving millions of lives during the Cholera pandemic. I express my sincere appreciation to honor and inspire her significant contributions to the field, which have served as a guiding light for many.

I'd like to thank Prof. Dr.Sassan Rezaie at Tehran University of Medical science , for his essential advice and assistance. In addition, I want to thank Professor Dr. Ali Akbari Sari for his support and contributions to my professional development. Thanks to Professor Annette Moter and her

group and Dr. Judith kikhney at Charité Medical University for their invaluable guidance and support, especially during the challenging times of the COVID-19 crisis.

Special thanks to Dr. Charlotte Rafaluk-Mohr for her invaluable support and guidance. Her expertise, encouragement, and dedication have been instrumental in shaping my understanding and passion for the subject matter. I thanks to Dr. Dirk J. Mikolajewski for his guidance and support. I sincerely thank Alexandro Rodriguez and Christin Manthey for their exceptional guidance. I'd want to offer my heartfelt appreciation to Dr. Stefan Rummel for his great assistance. I'd also want to thank the Rolff group for creating an inspirational and enjoyable workplace.

I want to express my heartfelt gratitude to my father, especially for his strength and support during his battle with cancer. His courage and love were unwavering, providing comfort even in the face of adversity. I am deeply thankful for the precious moments we shared, and his enduring spirit continues to inspire me. Though he is no longer with us, his impact on my life will forever be cherished.

I'd to extend my deepest thanks to my mother. Her unwavering love, support, and sacrifices have shaped who I am today. I am truly grateful for her endless kindness and the countless ways she has enriched my life. Her presence is a source of strength and comfort, and I appreciate her more than words can convey.

Gratitude fills my heart as I extend my deepest thanks to my brothers. Their support, camaraderie, and shared experiences have been invaluable. I am thankful for the bond we share and for the strength and laughter, they bring into my life. Their presence makes every journey more meaningful, and I'm truly blessed to have them by my side.

PUBLICATIONS

Published

Noushin Arfatahery ,et al ,Characterization of Toxin Genes and Antimicrobial Susceptibility of *Staphylococcus aureus* Isolates in Fishery Products in Iran. *Scientific Reports* **6**, Article number: 34216(2016) doi:10.1038/srep34216 . https://www.nature.com/articles/srep34216

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In prep.

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